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Review

Analytical strategies for shotgun phosphoproteomics: Status and prospects

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ABSTRACT

New analytical strategies for phosphoproteomics, both experimental and computational, have been rapidly introduced in recent years, leading to novel biological findings on the role of protein phosphorylation, which have in turn stimulated further development of the analytical techniques. In this review, we describe the development of analytical strategies for LC–MS/MS-based phosphoproteomics, focusing particularly on recent progress in phosphopeptide enrichment, LC–MS/MS measurement and the subsequent computational analysis. High-coverage analysis of the phosphoproteome has largely been achieved by combining pre-fractionation methods with multiple phosphopeptide enrichment approaches, at some cost in LC–MS/MS measurement time and increased sample loss. Key points for the future will be to further increase the selectivity and the recovery of enrichment methods to achieve higher sensitivity and efficiency in LC–MS/MS analysis in order to detect protein phosphorylation comprehensively, including low-abundance proteins. This is expected to lead to a more detailed understanding of the mechanisms and interactions of phosphorylation-mediated regulatory pathways in biological systems.

Contents

1. Introduction

Protein phosphorylation by kinases is a post-translational modification that is commonly observed in a wide variety of organisms, from bacteria to human. Among amino acids that can be modified with phosphate, serine, threonine and tyrosine are by far

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the most commonly found. In cellular signal transduction pathways, the combination of kinase-induced phosphorylation and phosphatase-induced dephosphorylation works like a switch to activate or inactivate the functions of proteins constituting the signaling pathways, thereby modulating many functions, including cell growth, cell division, apoptosis and cell death. So, comprehensive acquisition of cellular phosphorylation data is crucial to understand cellular functions and mechanisms [1–3].

Though many proteins are phosphorylated by kinases, the abundance of phosphorylated proteins is generally low in cells, and this represents a bottleneck for comprehensive analysis of the phosphoproteome. Conventionally, antibody-based methods such as Western blotting have been used to analyze phosphorylation of proteins. However, these methods require a phosphorylation-specific antibody for each case. On the other hand, shotgun phosphoproteomics, which utilizes liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) together with highly selective phosphopeptide enrichment techniques, makes it feasible to identify tens of thousands of phosphorylation sites on proteins without specifying particular targets. This approach allows the discovery of novel phosphorylation sites, whereas antibody-based approaches require prior information on the target phosphorylated proteins.

Fig. 1 shows the workflow for shotgun phosphoproteomics. Protein mixtures are first digested into peptides, generally by cleavage with trypsin at the C-termini of Lys and Arg, and subsequently low-abundance phosphopeptides are enriched. These phosphopeptides are separated by reversed-phase LC and are directly subjected to tandem mass spectrometry (MS/MS). Since huge amounts of data are generated by LC–MS/MS analysis, the subsequent computational analysis is crucial for picking peaks from the raw MS data, identifying proteins from the detected peaks, and further systematic analysis. In this review, we focus on LC–MS/MS-based shotgun phosphoproteomics from the viewpoint of technology development in the fields of (1) phosphopeptide enrichment, (2) LC–MS/MS measurement and (3) computational analysis of MS data.

2. Phosphopeptide enrichment

2.1. Immobilized metal ion affinity chromatography (IMAC)

Immobilized metal ion affinity chromatography (IMAC) utilizes the affinity of metal ions for phosphate. Generally these metal ions are immobilized onto the chromatographic materials via a metal-chelating moiety. Phosphopeptide enrichment is carried out by (1) loading samples onto the column to trap phosphopeptides selectively, (2) washing the column to remove unphosphorylated peptides, and (3) eluting phosphopeptides at high pH or with phosphate salts. Andersson et al. first introduced Fe^{3+}-IMAC for phosphopeptides [4]. Since then, other metal ions such as Ga^{3+}, Zr^{4+}, Al^{3+}, Cu^{2+}, Ni^{2+} have been employed [5]. However, the selectivity is insufficient for application of IMAC approaches to large-scale phosphoproteome analysis of whole cell lysates, because acidic peptides having Glu and Asp also show affinity for IMAC beads. Therefore, methyl esterification of carboxylates [6], change of pH, and optimization of the concentration of organic solvent in the buffer [7–9] have been used to improve the specificity of purification, and have contributed to a great increase of IMAC selectivity. A recent study indicated that Ti^{4+} immobilized on polymer beads has a superior capability for phosphopeptide enrichment from whole cell lysates compared with IMAC using other metals or metal oxide affinity chromatography (MOAC; see below). After pre-fractionation by strong cation exchange (SCX) chromatography, Ti^{4+}-IMAC resulted in identification of 9000 unique phosphorylation sites from 400 μg of triple dimethyl labeled MCF-7 digest [10,11]. An alternative method based on interaction between metal ion and phosphopeptide is phosphate precipitation with Ca^{2+} and Ba^{2+} [12,13]. Apart from methods employing immobilized agents, a crystalline form of calcium phosphate, hydroxyapatite (HAP), has also been used to enrich phosphopeptides [14,15].

2.2. Metal oxide affinity chromatography (MOAC)

Phosphopeptide enrichment using MOAC was first introduced in 1997 by Ikegami and Nakamura, who employed titanium dioxide to enrich partially hydrolyzed phosphoproteins, i.e., phosphopeptides [16]. Other groups subsequently applied this method in phosphoproteomics studies [17–20]. However, TiO_2-based MOAC suffers from low specificity due to the competitive binding of acidic residues on non-phosphopeptides [21]. To overcome the problems of TiO_2-based MOAC, benzoic acid derivatives such as 2,5-hydroxybenzoic acid (DHB) or phthalic acid were used as competitive additives [22]. However, these aromatic acids were too hydrophobic to be removed by the desalting step before LC–MS/MSMS analysis. Sugiyama et al. developed an aliphatic hydroxy acid-modified metal oxide chromatography (HAMMOC) [23], in which an aliphatic hydroxy acid, such as lactic acid, and phosphate anion form cyclic chelates with one and two units of TiO_2, respectively (Fig. 1). The order of affinity for TiO_2 is phosphate groups first, followed by lactic acid, while the carboxylic groups of acidic amino acid residues show the weakest affinity. When the TiO_2 beads are blocked with lactic acid first, phosphopeptides in the subsequent loading step compete out lactic acid for TiO_2 binding, while acidic non-phosphopeptides remain unbound because their affinity for TiO_2 is weaker than that of lactic acid. The HAMMOC approach drastically reduces the non-specific binding of acidic non-phosphorylated peptides to TiO_2 and allows enrichment of phosphopeptides directly from complex cell lysates without prefractionation, in contrast to other phosphopeptide enrichment methods, such as IMAC and DHB/phthalate–titanium dioxide chromatography [22,24]. In addition, aliphatic hydroxy acids can be easily removed by desalting with reversed-phase cartridges, which is necessary for subsequent LC–MS/MS analyses. These findings were later confirmed by another group, who used glycolic acid instead of their original DHB/phthalate methods [25].

Other metal oxides have been used, including ZrO_2 [26], AlO_3 [27], Nb_2O_5 [28], SnO_2 [29], HfO_2 [30], and Ta_2O_2 [31], but TiO_2 is still the most widely used. Both multiply and singly phosphorylated peptides bind to TiO_2. Singly phosphorylated peptides are eluted with a typical eluent (pH 10–11.5), but there are several reports indicating that multiply phosphorylated peptides are also eluted under different pH conditions [32,33].

Several systematic schemes have been proposed for TiO_2 enrichment. Pipette tip-based off-line TiO_2 mini-columns have been widely used for phosphopeptide purification [34,35]. A 2D-LC–MS/MS method employed TiO_2 as the first dimension and reversed-phase material as the second dimension [36,37]. Microfluidic HPLC-Chip/MS was also applied for TiO_2-based phosphoproteome enrichment [38].

2.3. Antibody-based enrichment for tyrosine phosphorylation

Although tyrosine phosphorylation (pTyr) is encountered less frequently than serine or threonine phosphorylation (pSer or pThr), it has a critical role in intercellular signaling mechanisms [39]. As antibodies for pSer and pThr have only limited specificity, pTyr antibodies have been mainly used for both enrichment by immunoprecipitation (IP) and detection by Western blotting [40–42]. The disadvantage of antibody-based methods is their biased preference depending on epitope recognition in the peptide sequences and the need substantial amounts of starting materials, so that challenging

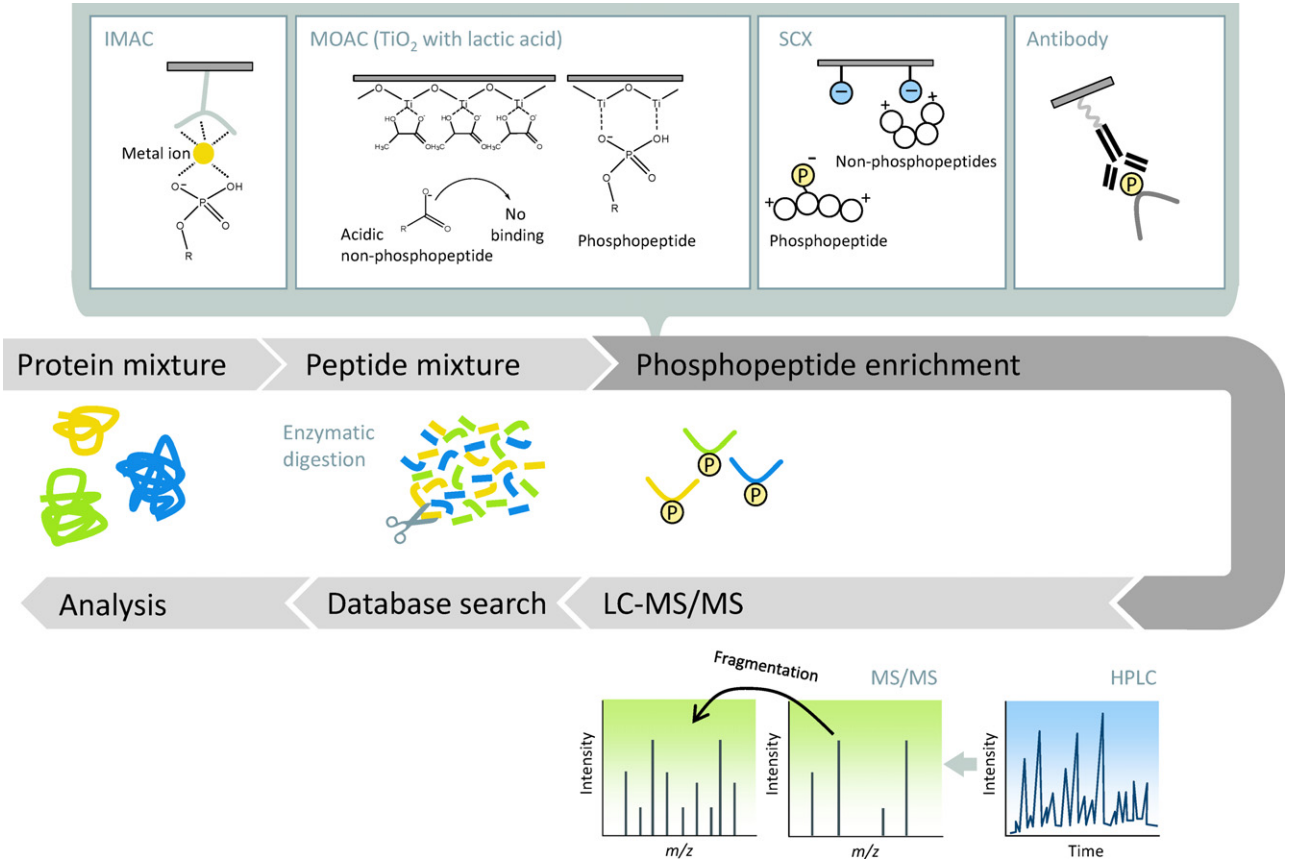

Fig. 1. Overview of shotgun phosphoproteome analysis.

large-scale purification is necessary. Nevertheless, in a recent study, Kettenbach et al. identified 3168 unique phosphotyrosine peptides from 8 mg of stimulated HeLa cell peptides using pTyr antibody for MOAC-enriched samples [43].

2.4. Ion exchange chromatography

Under an acidic condition (~pH 2.7), tryptic non-phosphopeptides possess a net charge of at least 2+ because of protonation of the N-terminal amino group and C-terminal Arg or Lys. However, negatively charged phosphate lowers the charge by one. Ion exchange chromatographic methods, i.e., strong cation exchange (SCX) with cation analyte or strong anion exchange (SAX) with anion analyte, are based on these differences in the solution charge states of peptides with or without a phosphate group. Using SCX alone, more than 2000 phosphorylation sites were identified from 300 μg of the nuclear fraction of HeLa cells [44]. But, peptides that have net zero or even negative charge, such as phosphopeptides with basic residues or multiply phosphorylated peptides, are not well retained on SCX columns. In order to capture these peptides, ultra acidic strong cation exchange was recently introduced [45], in which tandem SCX is performed under two different pH conditions (usual and more acidic conditions). Furthermore, based on a comparison among Lys-N, Lys-C and trypsin, Gauci et al. proposed Lys-N as a complement to trypsin to obtain a greater variety of fraction profiles in SCX [46].

2.5. Hydrophilic interaction chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) fractionates biomolecules based on their polarity (hydrophilicity). Those molecules are retained either weakly or not at all on reverse-phase columns, which are usually used for phosphoproteomic analysis. Samples are loaded in a high organic solvent concentration and eluted with a gradient of an aqueous solvent. The strong hydrophilicity of the phosphate group results an increase in retention time compared with non-phosphopeptides [47]. Recently, HILIC was applied to large-scale analysis and allowed the identification of more than 1000 phosphorylated sites from 300 μg of HeLa cell lysate, in combination of IMAC [48]. Similar to HILIC, electrostatic repulsion hydrophilic interaction chromatography (ERLIC) on a weak anion exchange (WAX) column has also been evaluated [49]. At low pH, carboxyl groups of Glu and Asp and the C-terminus are largely protonated and peptides with positively charged N-termini are electrostatically repelled from the column. However, negatively charged phosphate groups of phosphopeptides interact electrostatically with WAX and their retention times are increased compared with non-phosphopeptides [50].

2.6. Prospects for enrichment methods

The current gold standard for in-depth analysis (i.e., obtaining a large segment of the phosphoproteome) is a two-step process consisting of fractionation using SCX or HILIC followed by enrichment using IMAC or TiO_2-MOAC. However, as each method is known to cover different kinds of phosphopeptides with only partial overlap [21], sequential elution from IMAC (SiMAC), involving IMAC enrichment prior to TiO_2-MOAC, was developed [51]. This method enables the separation of mono-phosphorylated peptides (by TiO_2) and multiply phosphorylated peptides (by IMAC) and provided a 3-fold increase in recovery of multiply phosphorylated peptides. Similarly, optimizing the IMAC protocol in a tandem process (IMAC–IMAC) has enabled separation of mono-phosphorylated and multiply phosphorylated peptides [52].

However, as the number of experimental processes is increased, loss of samples can become a serious issue. For instance, by omitting the process of injection by autosampler, Masuda et al. achieved an approximately 80-fold improvement of sensitivity, resulting the identification of hundreds of phosphopeptides from 1 μg protein, consuming only 10,000 cells [53]. Even though fractionation is a reasonable strategy to reduce the complexity of biological samples, there is a tradeoff of MS instrument time and sample amount, which may be critical especially for clinical samples [54]. The key to achieving a comprehensive "one-shot phosphoproteome" method to cover the whole phosphoproteome in a single MS analysis is the improvement of both sensitivity and specificity. For example, improvement of peptide separation by LC resulted in an increase in the number of identified peptides [55,56]. This method should be applicable for phosphoproteome analysis to obtain greater numbers of identified phosphopeptides.

3. Phosphoproteome analysis by LC–MS/MS

3.1. Adsorption of phosphopeptides on LC–MS systems

Since the phosphate group has affinity to metal materials, which are typically used for LC–MS/MS as connectors, tubings, valves and so on, phosphopeptides, especially multiply phosphorylated peptides, are often recovered very poorly. Also, these acidic peptides tend to have lower ionization efficiency of the electrospray process in positive-ion-mode LC–MS/MS. A chemical reaction was developed to specifically transform pSer and pThr by β-elimination [57,58]. Phosphoric acid was reported to be effective as an additive to increase the recovery [59]. Ethylenediaminetetraacetic acid (EDTA) is another candidate as an additive to increase the number of identified phosphopeptides, but it has been reported to cause clogging and destruction of the spray needle and analytical columns due to precipitation [60,61]. Citrate was selected as an alternative additive and proved to enhance phosphopeptide identification similarly to EDTA [62].

3.2. Fragmentation methods

After a digested peptide is injected into the MS, a precursor ion is fragmented into product ions. The abundance and richness of fragmentation ions are important factors for the effective identification of phosphorylated sites in shotgun proteomics. Technological development in this area has recently been very rapid, and very powerful MS instruments have become available.

3.2.1. Collision-induced dissociation (CID) by ion trap

Collision-induced dissociation (CID) is a standard fragmentation technique in proteomics and phosphoproteomics. In CID, protonated peptides are accelerated by an electrical potential in the vacuum chamber of the mass spectrometer. Then a neutral gas (e.g., helium, nitrogen or argon) is introduced and bond disruption occurs to generate a series of b- and y-ions [63]. Even with low-energy CID (less than 100 eV), the *O*-phosphate bonds in serine- and threonine-phosphorylated peptides are labile during this process, and neutral loss (elimination of phosphate) of phosphopeptides tends to dominate over dissociation of the main peptide backbone. To prevent or minimize neutral loss, pseudo-MS^3 [64] or neutral loss directed MS^3 [65] has been developed. In those strategies, product ions generated by neutral loss are again fragmented to cleave the peptide backbone. A possible issue in CID is intermolecular phosphate transfer reaction in the ion trap. Aguiar et al. used synthetic peptides to examine this issue, and found that phosphate transfer does occur, but only doubly charged precursors form measurable amounts of transferred fragments. Since only a part of the ions undergoes the reaction, there is no critical effect on the precision of site determination [66].

3.2.2. Electron capture dissociation (ECD)/electron transfer process (ETD)

Electron capture dissociation (ECD) [67] or the related electron transfer dissociation (ETD) [68,69] was developed as a gentler fragmentation technique than CID. Both technologies provide similar fragmentation patterns, with the generation of c- and z-ions, and no loss of phosphorylations [68,70,71]. This technology has been applied to proteome and phosphoproteome studies [72–74]. ETD and CID can be regarded as complementary strategies, though ETD is advantageous for site determination [75–77]. These methods become more efficient as the ratio of charge to number of amino acid residues in a target peptide is increased. Trypsin, which is generally utilized for protein digestion, mainly produces 2+ peptides, which have been shown to be suboptimal for ETD fragmentation [74–76,78]. Switching the location of the basic residues from the C-terminal to the N-terminal side of the peptide improves the coverage of peptide sequencing by ETD. In order to generate suitable digested peptides, an enzyme called Lys-N has been utilized in combination with ETD. Several alternative proteases, such as Lys-C, Glu-C and chymotrypsin, have also been used in ETD analysis, in addition to Lys-N [76,79]. Swaney et al. proposed a decision-tree-based data-dependent MS approach in which either CID or ETD was used for sequencing depending on the characteristics of the targeted peptides [78].

3.2.3. Higher-energy collisional dissociation (HCD)

Higher-energy collisional dissociation (HCD) was firstly introduced as higher-energy C-trap dissociation [80]. The collision mechanism of HCD is basically the same as that of CID in triple quadrupole or Q-TOF type MS, and the fragmentation patterns are similar to those obtained with CID, i.e., b- and y-ions are generated. Because of its ability to detect low-mass fragment ions, HCD is utilized for isobaric tag-based quantitation (e.g., iTRAQ) [81,82], and for pTyr determination using immonium ion. Due to reduced intensity of the neutral loss peak at higher collision energy, HCD produce clearer fragment ion spectra for pSer- and pThr-containing peptides [80].

4. Computational analysis

4.1. Phosphorylation site localization

The assignment of phosphorylation sites within the peptide sequence is not necessarily easy, especially in cases where multiple phosphorylation sites exist side-by-side on a peptide. Several software tools have been developed for automatic evaluation of the reliability of phosphorylation site identification, using Ascore or PTM score [83,84]. Both are based on a comparison of the calculated probability of the fragment occurrence with the experimentally obtained spectrum. SIDIC (site-determining ion combination) adapts basically the same idea, but considers the case that the fragment ion may contain multiple possible phosphorylation sites and adopts that case as an identification of ambiguous phosphorylation sites, which are rejected in the other two algorithms [85]. These algorithms are for b- and y-ion spectra, which are generated in CID, but site determination software for ECD/ETD to deal with c- and z-ions has also been developed [86].

4.2. Public phosphorylation data and prediction tools

With the exponential increase in the number of identified phosphorylation sites, comprehensive phosphorylation databases that contain qualitative and quantitative information have become

available as an information source for systems biology; these include Uniprot [87], PhosphoSitePlus [88], Phospho.ELM [89], and Phosida [90]. These databases include not only basic protein and modification information, but also related information such as structure, localization, motif sequence, and substrates. Each database has unique features in this regard. Database like human protein reference database (HPRD) [91] and NetPhorest [92] collects motif sequences of kinases.

Although MS-based analysis is good at identification of large numbers of substrates, the upstream kinase is not detectable. But, the motif sequence can be used to predict the upstream kinase from the substrate sequences using Scansite 2.0 [93], KinasePhos2.0 [94], GPS2.0 [95], NetPhosK [96], PREDIKIN [97], and NetPhorest [92]. In order to predict kinase-substrate pairing, NetworKIN [98] uses not only motif information, but also additional comprehensive datasets, such as protein-protein interaction from STRING, co-localization, transcriptome data, and co-mentions in the literature. This integrated analysis approach has improved the prediction accuracy of NertworKIN by 2.5-fold as compared to motif-based prediction alone. Motif-X [99] is a web application to extract motif sequences from substrate sequences by calculating the occurrences of amino acids around phosphorylation sites. PhosphoSitePlus [88] also provides a motif analysis tool, which is a modification of the ScanProsite tool [100].

5. Prospects

Led by technological improvements of MS and increased sophistication of enrichment strategies, high-throughput detection of phosphorylation sites has become feasible, and the phosphoproteome is being explored. Among enrichment methods presently available, each has a certain preference or bias within phosphopeptides, so the use of combinations of enrichment methods is a promising approach to achieve high-coverage proteome detection. However, two-step strategies using fractionation and combinations of multiple enrichment strategies require a lot of MS instrument time and increase the likelihood of sample loss, particularly for low-abundance proteins, during the multiple enrichment processes. Therefore, the key to whole phosphoproteome detection by MS analysis will be the development of simple enrichment processes that provide increased selectivity for phosphopeptides while maintaining high coverage. Complementary to this will be the further development of MS instruments to allow more sensitive and specific detection of the phosphoproteome by increasing both the detection sensitivity and the scan speed of ion detection [101]. Such analytical methods should also be useful for proteomics, and comparisons of proteome and phosphoproteome should throw light on the functional meanings of phosphorylation stoichiometry. Improved computational analysis of very large phosphorylation datasets will also be important for the interpretation of the functional significance of phosphorylation in biological regulation, especially where multiple phosphorylations occur cooperatively and are integrated into specific regulatory pathways. Incorporation of other omics datasets, including post-transcriptional modification datasets, will also be required to understand in detail the functions and integration of regulatory pathways.

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