Analysis of protein phosphorylation by mass spectrometry

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Abstract

The reversible phosphorylation of proteins is recognized as an essential post-translational modification regulating cell signaling and ultimately function of biological systems. Detection of phosphopeptides and localization of phosphorylation sites remains quite a challenge, even if the protein is purified to near homogeneity. Mass spectrometry has become a vital technique that is routinely utilized for the identification of proteins from whole cell lysates. Nonetheless, due to the minimal amount of phosphorylation found on proteins, enrichment steps for isolating phosphopeptides from complex mixtures have been the focus of many research groups worldwide. In this review, we describe some current methods for the enrichment of phosphopeptides that are compatible with mass spectrometry for assignment of phosphorylation sites. Phosphorylation modifications on proteins and peptides are either directly isolated by solid-phase approaches or chemically modified for selective isolation and/or improved characterization by mass spectrometry. These strategies hold the potential for rapid and sensitive profiling of phosphoproteins from a variety of sources and cellular conditions.

Keywords: Phosphorylation; Post-translational modification; Mass spectrometry; IMAC; MS/MS; β-Elimination; Phosphopeptide; Phosphoprotein

1. Introduction

Mass spectrometry based proteomics research is in part driven by the fact that the post-translational modification properties of proteins cannot be determined from DNA sequence alone. One of the most common and important modification of proteins comes in the form of phosphorylation to serine, threonine or tyrosine residues. Protein phosphorylation has been known to be a significant regulatory mechanism in many organisms controlling a wide variety of biological functions [1–4]. In fact, nearly 2% (>2000 genes) of the human genome encode for protein kinases, with an estimated 30% of all proteins thought to exist in phosphorylated forms [2]. Nevertheless, despite phosphorylation being acknowledged as a crucial modification involved in many cellular events, determining the sites of phosphorylation on proteins is not a routine task.

Traditional methods for the analysis of phosphoproteins include radioactive labeling of the protein with 32P to monitor phosphorylation, and Edman degradation chemistry on phosphopeptides to localize the site of phosphorylation [5]. Although some success has been reported with those methods, these techniques have disadvantages of being relatively time consuming and laborious, requiring large amounts of purified protein and Edman degradation does not work on proteins/peptides with blocked N-termini (i.e., N-terminal acetylation). Recently, mass spectrometry based methods have emerged as powerful and preferred tools for the analysis of post-translational modifications including phosphorylation due to higher sensitivity, selectively, and speed than most biochemical techniques [6–8].

Mass spectrometry approaches for the analysis of phosphorylation sites have mostly relied on using an
instrument capable of tandem mass spectrometry (MS/MS) experiments to determine the sequence and location of phosphorylation sites on peptides. However, despite the advances in mass spectrometry identification of phosphopeptides, some difficulties still remain. First, the stoichiometric level of phosphorylation for a given protein may be very low. Second, the signals in the mass spectrometer due to a phosphopeptide have also been observed to be lower than its non-phosphorylated version. Lastly, the MS/MS properties of phosphopeptides undergoing low energy collisionally activated dissociated (CAD) fragmentation are in most cases challenging to interpret.

For these reasons, it is to the researchers benefit to separate the phosphorylated peptides/proteins in mixtures from non-phosphorylated species to improve the detection and identification of phosphorylation sites. Phosphotyrosine antibodies have been previously employed with some success, although immunoprecipitation using phosphoserine and phosphothreonine antibodies are not quite yet available [9]. Over the last several years, there have been large amounts of literature that have focused on the improvement of phosphopeptide characterization using mass spectrometry [10–17]. Therefore, any efforts to comprehensively summarize and report the current state of knowledge and techniques in this area will likely fall short in one aspect or another. In this report, we have detailed three general methods for enhanced analysis of phosphopeptides by mass spectrometry, as well as highlighting some information and potential challenges involved in the identification of phosphorylation sites for beginning mass spectrometry users.

2. Description of methods

2.1. Experimental design and sample preparation

To maximize phosphorylation levels on the protein(s) of interest, biological preparation of a sample should include using phosphatase and protease inhibitors in the cell lysis buffer to minimize de-phosphorylation and degradation of the phosphoprotein. Usually, 2 mM sodium vanadate or 50 μM calyculin and protease inhibitors 2.5 μg/mL leupeptin, 2.5 μg/mL pepstatin, 2.5 μg/mL aprotinin, 2.5 μg/mL antipain, and 2.5 μg/mL chymostatin can be included in the cell lysis buffer for preparation of the proteins. The initial step in phosphoprotein site mapping is to enzymatically digest the protein sample with a protease such as trypsin (other proteases can be utilized depending on the protein sequence and desired coverage) to produce a mixture of smaller peptides that are of sufficient length for fragmentation in a mass spectrometer. Next, since the amount of phosphorylation of proteins can occur at relatively low levels, it is advantageous to enrich for phosphopeptides by using a solid-phase method which can bind the phosphopeptides, and where washings could be employed to remove non-phosphorylated fragments. The remaining phosphorylated peptides can be introduced into the mass spectrometer via liquid chromatography coupled to electrospray ionization (ESI), or by using matrix-assisted laser/desorption ionization (MALDI). Tandem mass spectrometry (MS/MS) experiments are typically utilized to produce fragmentation spectra which can be searched using various programs such as Sequest or Mascot against the selected database or manually interpreted to determine the site of phosphorylation on the peptide. Search parameters typically include using a differential modification of +80 Da on serine, threonine, and tyrosine residues along with a static modification of +14 Da (methyl groups) on aspartic and glutamic acid residues and on the C-terminus of each peptide, if the peptide digests were converted to methyl esters prior to MS analysis (see Section 2.2). Other modifications to amino acid residues can also be included in the search parameters, such as oxidized methionine (+16 Da), etc. Results of any database search should always be manually inspected to confirm the modification site on the peptide. In some cases, taking an MS/MS spectrum of a synthetic peptide corresponding to the phosphopeptide in question may aid in localization of phosphorylation sites as well.

2.2. Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) (first named metal chelate affinity chromatography) was first developed by Andersson and Porath [10] and successfully applied for the separation of several types of proteins by the use of various chelated metals. Immobilized iron (III) ions have been shown to interact with the phosphate groups present on phosphoproteins and used to selectively isolate phosphopeptides from digestion mixtures, shown in Fig. 1. Previously, it has been observed by our group and others that non-phosphorylated peptides containing acidic residues also bind and are retained on IMAC columns. To prevent non-specific binding of non-phosphorylated peptides to the IMAC columns, all peptides can be converted to corresponding methyl esters [11]. It is important that all solvents from the peptide solutions be evaporated before the addition of the esterification reagent to obtain complete conversion of carboxylic groups to methyl esters and reduce side products. The reagent for the esterification reaction is made by the addition of 160 μL acetyl chloride to 1 mL methanol (2 M methanolic HCl solution). A 100 μL aliquot of this reagent solution is added to 50 pmol (or less) peptide material and allowed to stand at room temperature for 1 h. The solvent is removed by lyophilization and the procedure is repeated. Peptide methyl esters are lyophilized to remove solvent a second time and redis-
solved in equal parts of methanol, acetonitrile, and 0.1% acetic acid for loading onto the IMAC column.

IMAC columns can be constructed by packing a fritted 360 µm o.d. × 100 µm i.d. fused-silica capillary column (Polymicro Technologies, Phoenix, AZ) with 8–10 cm of POROS 20 MC resin (Applied Biosystems, Framingham, MA). A series of rinses on the IMAC column are then performed to prepare and activate the columns. Columns are first washed with 50 mM EDTA (pH 9.0) for 10 min at a flow rate of 20 µL/min to extract free metal ions from the column. A wash with Nanopure deionized water (pH 7) for 5 min (20 µL/min) is then used. Note that this wash step is utilized to neutralize the pH as addition of FeCl₃ without this wash step could produce iron hydroxide precipitation. The columns are next activated with 100 mM FeCl₃ for 10 min (20 µL/min). Excess non-chelated Fe³⁺ ions are removed by washing the column with approximately 20 µL of 0.1% acetic acid. Peptide methyl ester samples are loaded onto activated IMAC columns at a low flow rate (<10 µL/min). Sometimes samples require a cleaning/desalting step prior to loading onto the IMAC columns, such as in the case for solutions containing excess ATP (from in vitro phosphorylation reactions), or samples previously dissolved in strong ionic solutions (i.e., urea). In these cases, the sample is first loaded onto a clean-up column consisting of packing a fritted 360 µm o.d. × 75 µm i.d. fused-silica capillary column (Polymicro Technologies, Phoenix, AZ) with 4–6 cm of 5–20 µm C₁₈ resin (YMC, Wilmington, NC). The column is then rinsed with 0.1% acetic acid for 10 min (20 µL/min). The peptides retained on the C₁₈ column can be eluted with 30 µL of 40% acetonitrile in 0.1% acetic acid and loaded onto an IMAC column connected to the C₁₈ column with 2 cm of

Fig. 1. IMAC enrichment of a phosphopeptide combined with a tryptic digest after blocking of carboxylic groups by methyl ester conversion with analysis by LC–MS/MS. (A) Selected-ion chromatogram versus scan number for m/z 564.5 ([M + 2H]²⁺ of the phosphopeptide DRVpYIHPF). (B) MS/MS spectrum of the DRVpYIHPF peptide at m/z 564.5 from scans 610 to 616. (C) ESI-MS recorded during the time when the DRVpYIHPF peptide eluted from the chromatographic run (note many non-phosphorylated peptides are present from non-specific binding to the IMAC column at much higher intensities than the ion at m/z 564.5). (D) SIC for m/z 578.5 corresponding to the dimethyl ester of the doubly charged DRVpYIHPF peptide. (E) MS/MS spectrum of the dimethyl ester of the [M + 2H]²⁺ ion of the phosphopeptide DRVpYIHPF eluting in scans 151–163. (F) ESI-MS recorded during the time when the dimethyl ester of the DRVpYIHPF peptide eluted from the chromatographic run. Now the dimethyl ester of the DRVpYIHPF ion is the most intense ion after removal of the non-phosphorylated peptides. Reprinted with permission from [11].
Teflon tubing. Once the sample is loaded onto the IMAC column, a rinse step with 15 μL of 0.01% acetic acid is employed. For complex samples where a clean-up column was not used, but that contain a large amount of non-phosphorylated contaminants (i.e., from whole cell extracts), an optional rinse step of 25 μL of a solution containing 100 mM NaCl and 25% acetonitrile in 0.1% acetic acid can be used. The IMAC column is connected to a 360 μm o.d. × 75 μm i.d. fused-silica capillary column packed with 4–6 cm of 5–20 μm C18 resin, and the captured phosphopeptides are eluted off the IMAC column using 15 μL of a 250 mM Na2HPO4 solution (pH 6). Phosphopeptides on the C18 column are washed with 0.1% acetic acid before the column is connected to an analytical column and gradient eluted into an LC-electrospray mass spectrometer. Phosphopeptides can also be collected after elution from the IMAC column and co-crystallized with a MALDI matrix for ionization into a time-of-flight (TOF) instrument.

2.3. Phosphopeptide isolation by chemical transformation methods

Chemical replacement of the phosphate group by an affinity tag or group that is more compatible for mass spectrometry analysis has been attempted in several reports [12–16]. One of the most common procedures involves using strong alkaline conditions to induce the phosphate group (on phosphoserine and phosphothreonine residues) to undergo β-elimination and form a more reactive dehydroalanine species. This residue can then act as a Michael acceptor and react with a selected nucleophile (Scheme 1, thiol based nucleophiles are most common). It is important to treat the protein sample with performic acid to oxidize cysteine residues prior to β-elimination, if reduction and alkylation of the protein was not first performed. Performic acid reagent can be created by adding 0.5 mL of 30% hydrogen peroxide to 4.5 mL of 88% formic acid (Wako Pure Chemicals, Osaka, Japan) containing 25 mg phenol. The solution is created by adding 0.5 mL of 30% hydrogen peroxide to a 250 mM Na2HPO4 solution (pH 6). It is important to treat the protein sample with performic acid to oxidize cysteine residues prior to β-elimination, if reduction and alkylation of the protein was not first performed. Performic acid reagent can be created by adding 0.5 mL of 30% hydrogen peroxide to 4.5 mL of 88% formic acid (Wako Pure Chemicals, Osaka, Japan) containing 25 mg phenol. The solution is then cooled to 4 °C and 0.5 mL of the reagent can be added to 5 mg of dried protein and reacted overnight at 4 °C followed by drying with vacuum evaporation.

Several variations of the conditions for β-elimination have been reported, but in general β-elimination reaction mixture can be prepared by adding 140 μL of 2–4 M LiOH (NaOH or Ba(OH)2 have also been utilized) to a solution of 50 μL acetonitrile including 100 mM Ba(NO3)2, EDTA (250 mM, pH 12) should be also added to the above reaction mixture to avoid side reactions (discussed in Section 2.6). The nucleophile that is to be used in the Michael addition can be included in the above mixture for a one-pot reaction scheme. Some common nucleophiles used include dithiothreitol (DTT) and 1,2-ethanedithiol (EDT). A 20 μL aliquot of EDT or DTT is diluted into 50 μL ethanol and added to the above reaction mixture. This reaction mixture is sufficient on protein amounts ranging from 10 to 100 μg. The reaction should proceed at 37 °C for 1 h and is neutralized with acetic acid. Although the conversion of phosphate groups to more stable thiol based derivatives has been observed to enhance the tandem mass spectra of phosphopeptides and thus their characterization, the coupling of the thiol groups to an affinity resin for the isolation of phosphopeptides provides a practical method for their specific selection.

Chait and co-worker [16] have developed two procedures for purification of phosphopeptides using β-elimination in combination with affinity columns based on activated thiol and biotin–avidin resins. For activating thiol resins, the above reaction mixture must be equilibrated with buffer solution (2.4% Chaps, 50 mM Tris–HCl, and 1 mM EDTA, pH 8.0) and added to activated thiol–Sepharose 4B affinity resin (Sigma Chemical, Milwaukee, WI) prepared by manufacturer’s instructions that was washed with the same above buffer solution. A 1 μL aliquot of bed volume of resin is used for every microgram of protein and incubated for 1 h with subtle mixing at room temperature. Non-specifically bound protein can be removed by washing first with the same buffer solution and second with a urea based buffer (8 M urea, 50 mM Tris–HCl, and 1 mM EDTA, pH 8.0). Proteins bound to the thiol–Sepharose beads can be digested directly with trypsin (37 °C for 4 h to overnight) after equilibrating the beads in 50 mM NH4HCO3 and unbound peptides were removed by washing with both

![Scheme 1](image-url)
NH₄HCO₃ and urea buffer solutions. Peptides bound to the beads can be eluted with the use of a reducing buffer containing 10 mM DTT in 50 mM NH₄HCO₃ (mixing 30 min at room temperature) and acidified for direct MS analysis.

Biotinylation enrichment of phosphoproteins can also be achieved from the above transformation of the phosphate group to a thiol derivative with DTT or EDT. After the protein is modified with the thiol functional group, it can be precipitated with 1 mL of ice-cold acetone, washing with ethanol, and centrifuged (20,000g) for 3 min followed by being redissolved in a 0.1 M sodium phosphate solution (pH 6.8) containing 8 M urea and 0.5% Chaps. About 5 mg of (+)-biotinyl,3-maleimido-3,6-dioxoctanediamine (Pierce Chemical, Rockford, IL) is added and the mixture is twice dialyzed against 3 L of 50 mM ammonium bicarbonate buffer solution. The biotinylated proteins can then be enzymatically digested and solution passed through an immobilized NeutrAvidin column (Pierce Chemical, Rockford, IL) followed by washing with 0.1 M sodium phosphate buffer solution containing 2% Chaps and 150 mM NaCl, and then with deionized water. The biotinylated peptides are eluted off the beads with 200 µL of an acetonitrile:water:TFA mixture (50:50:1), dried down, and brought up in a solvent compatible for MS analysis (i.e., 0.1% acetic acid or 0.1% aqueous TFA).

2.4. Phosphospecific proteolysis

An extended version of the β-elimination protocol to convert phosphoserine and phosphothreonine to dehydroalanine and β-methyldehydroalanine, respectively, under basic conditions and then to produce a derivative that can be specifically targeted for proteolytic digestion has been developed by Shokat and co-workers [17]. The goal of this strategy was to make an aminoethylcysteine (or phosphothreonine, β-methylaminoethylcysteine is generated) moiety that is isosteric with lysine and can be cleaved specifically by proteases that recognize lysine residues such as Lys-C and trypsin (Scheme 1, R = NH₃). Therefore, phosphorylation sites would always be located on the C-terminal residue of a peptide and also is recognizable by a unique y₁ ion in MS/MS spectra (Fig. 2), thus facilitating interpretation of phosphopeptide tandem mass spectra. After β-elimination is performed on the protein sample (using similar conditions described previously), 1 M cysteamine in water (50 µL) is added to the reaction mixture and allowed to incubate at room temperature for 3–6 h. Digestion buffer consisting

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Fig. 2. ESI-MS/MS of the [M + 2H]²⁺ ion at m/z 886.40 of a β-casein phosphopeptide with phosphoserine modified to aminoethylcysteine. The aminoethylcysteine-modified β-casein was digested with trypsin/Lys-C prior to MS analysis. Note that K* represents aminoethylcysteine. Reprinted with permission from [17].
of 10 mM Tris, pH 8.5, for trypsin or 10 mM Tris, pH 8.5, 1 mM EDTA for Lys-C is added to the sample and digested overnight at 37 °C. After acidification, the solution is ready for mass spectrometry analysis. A solid-phase approach for this reaction sequence has been also developed for the isolation of phosphopeptides from complex mixtures.

2.5. Instrumentation

As the goal of mapping phosphorylation sites was to localize the sites on specific threonine, serine or tyrosine residues on peptides, a mass spectrometer that is capable of producing tandem mass spectrometry fragmentation spectra should be utilized. Such instruments include, but are not limited to, ion trap and quadrupole time-of-flight mass spectrometers. Currently, the quadrupole ion traps (LCQ, LCQ XP or LTQ, ThermoElectron, San Jose, CA) with electrospray ionization are the workhorse instruments in our laboratory. These mass spectrometers are very robust, easily interfaced with LC instruments for online chromatography, and require minor maintenance. LC-ESI is especially attractive since different phosphorylation sites can exist on the same peptide and although these peptides might elute at similar times on a C18 column, resolution of the peaks may be possible by reverse-phase HPLC. The ion traps can be operated in a data-dependent setting allowing a full-scan mass spectrum along with 5 MS/MS spectra to be recorded sequentially of the five most abundant ions in the full mass spectrum. These ions are then placed on an elution list to avoid recording redundant data, and the cycle is repeated (about every 12 s). This method allows for increased sensitivity with the routine analysis of peptides in the low femtomolar concentration with a dynamic range of approximately 100:1. Instruments that do not provide sequence information could possibly be used for phosphopeptide site analysis, but the peptide identified by mapping must contain a sole phosphate receptor residue (only one serine, threonine or tyrosine) for unambiguous assignment of the phosphorylation site.

2.6. Data analysis

A typical time-line for completion of the phosphopeptide mass spectrometry based analysis is approximately several days to one week. Usually, two to three days are spent on the sample preparation including enzymatic digestion, any chemical modification, and instrument time. The database searching can take 30 min to a couple of days to complete depending on the number of files to be searched, the size of the database, and computing power. Nevertheless, the bottleneck of the process lies in the manual validation of the MS/MS spectra. It is our experience that all phosphopeptide MS/MS spectra should be manually confirmed, especially if the phosphorylation site determined by the computer search is located on a peptide containing multiple potential sites of phosphorylation. MS users should expect to spend one to several days manually confirming all phosphopeptide MS/MS data. In addition, it is in some cases a good idea to synthesize the suspected phosphopeptide for comparison of the MS/MS spectra against the spectra generated from the true biological sample. The interpretation of phosphopeptide MS/MS spectra is complicated by the fact that most phosphorylated peptides typically display partial to full loss of H3PO4 (m=98) in their CAD spectra from the precursor as well as b and y type fragment ions. However, this characteristic can be used to distinguish and find phosphopeptide spectra or possibly used for precursor ion scanning. Fig. 3 shows the ESI-MS/MS spectrum taken on a LCQ ion trap instrument of a doubly charged precursor phosphopeptide ion from a tryptic digest of a propionylated histone H3 sample from asynchronously grown HeLa cells with the sequence K(PrMe)pSAPATGGVK(Pr)K (Pr)PHR (27–40 residues). This sample has been chemically derivatized with propionic anhydride to add a propionyl group (Pr) on endogenously unmodified and mono-methylated lysine residues to remove charge and improve hydrophobicity for improved MS analysis of histone derived peptides [18]. As can be seen in Fig. 3A, the most intense fragment ion results from the loss of H3PO4 to the precursor ion (−49 Da for a doubly charged species). At first glance, there does not seem to be a high amount of fragment ions produced for the positive identification of the phosphorylation site on this peptide. However, a closer inspection of the y series of ions near the baseline level (Fig. 3B, magnification of grass regions surrounding the [M + 2H – H3PO4]2+ ion) clearly indicates that not only is the phosphate group present on the serine residue, but that there also is a mono-methyl modification present on K27 and not on K36 or K37. The b series of ions are a little more difficult to detect since the phosphorylation site is near the amino-terminus and hence all b ions following the phosphoserine residue are capable of losing the phosphate moiety. Indeed, this is exactly what is observed on this phosphorylated histone peptide as a series of singly charged b ions with a loss of 98 Da can be seen in the MS/MS spectrum. Generally, it is necessary to inspect the ions in the baseline level to sequence phosphopeptide MS/MS spectra.

Since the level of phosphorylation on a protein can be of very low abundance, selectively separating the phosphopeptides from other non-phosphorylated peptides is almost a necessity. IMAC on fairly complex protein digest mixtures has been shown to be very effective in the isolation of phosphopeptides when combined with conversion of peptide carboxylic acid groups to the corresponding methyl esters. Fig. 1 demonstrates the importance of converting carboxylic groups on acidic
residues and C-termini to methyl esters to prevent binding of non-phosphorylated peptides to the IMAC column from a tryptic digest of several standard proteins (1 pmol each) combined with the synthetic phosphopeptide DRVpYIHPF (5 fmol). Fig. 1A shows the selected-ion chromatogram (SIC) for \( m/z \) 564.5 as a function of the scan number. In Fig. 1B, the ESI-MS/MS spectrum of an ion at \( m/z \) 564.5 eluting in scans 610–616 corresponding to the sequence DRVpYIHPF is displayed. A full electrospray mass spectrum acquired during this same time frame is shown in Fig. 1C. It should be noted that there are many non-phosphorylated signals in the mass spectrum and no signal above chemical noise for the phosphopeptide at \( m/z \) 546.5, therefore, it is concluded that non-phosphorylated tryptic peptides containing multiple acidic residues can efficiently bind to the IMAC column. To reduce this non-specific binding, the sample was treated with methanolic HCl to convert carboxylic groups to methyl esters. Fig. 1D shows the SIC for \( m/z \) 578.5 (double methyl ester of the peptide DRVpYIHPF), and its MS/MS spectrum is displayed in Fig. 1E. The electrospray full mass spectrum during the time period where this phosphopeptide dimethyl ester elutes is shown in Fig. 1F. Now the signal for the phosphopeptide \( m/z \) 578.5 is detectable and is the most abundant ion in the mass spectrum after removal of the
non-phosphorylated species. IMAC works well to isolate phosphopeptides from complex mixtures containing many more abundant non-phosphorylated peptides when all peptides are converted to the corresponding methyl esters. This methodology has been successfully applied for the identification of 216 peptide sequences containing over 383 sites of phosphorylation from a whole cell lysate of Saccharomyces cerevisiae [11]. Differential expression/quantification analyses are also possible as one sample can be converted to methyl esters with methanolic HCl, while a second sample could be isotopically labeled with deuterated methanolic HCl. The samples can be equally mixed and introduced into the mass spectrometer for relative quantitation of peptides obtained under different cellular conditions [19].

The β-elimination/Michael addition methods in Section 2.3 have been demonstrated to improve the MS analysis of phosphopeptides not only through enhanced MS/MS fragmentation, but also by coupling the reactions to solid-phase thiol–Sepharose or biotin–avidin affinity resins. The experimenter should be conscious of the expected mass shift of the peptide upon losing phosphate and incorporating a thiol tag (i.e., $-4\text{Da}$ for loss of $\text{H}_3\text{PO}_4$ and addition of EDT, $\text{C}_2\text{H}_7\text{H}_2\text{S}_2$). A useful second experiment would also be to use a phosphatase on the original sample for subtraction of background non-phosphorylated peptide masses from true modified peptides, as no change in mass would be expected from non-phosphorylated peptides after treatment with a phosphatase. It should be pointed out that McLachlin and Chait [16] reported that care should be taken when performing and analyzing the β-elimination reactions, since a small percentage of unphosphorylated serine residues was detected to be modified and co-purified with the phosphopeptides of interest. The authors report that the addition of EDTA and $\text{Ba(NO}_3)_2$ to the reaction mixture decreased the amount of side reaction at serine, but that this side reaction could not be completely eliminated. The thiol–Sepharose beads are also preferred to avidin beads, as inefficient recovery of peptides modified with a biotin tag can result due to the strong biotin–avidin interaction. Additionally, the maleimide linker on the biotin tagged peptide is also somewhat unstable to fragmentation in mass spectrometers causing fragmentation patterns that are not trivial to decipher.

Although the techniques mentioned above have been used to purify phosphorylated peptides, a direct technique that specifically cleaves phosphorylation sites on proteins has also been recently developed [17]. In the method mentioned in Section 2.4, β-elimination chemistry is used to produce a dehydroalanyl residue and the nucleophile used in the subsequent Michael addition reaction is cysteamine (Scheme 1, $R = \text{NH}_3$). This reaction produces a functional group that resembles a lysine residue (phosphoserine produces aminoethylcysteine which is isosteric with lysine) and can be digested with a protease that recognizes lysine residues such as trypsin or Lys-C. Phosphothreonine produces β-methylamino-cysteine which was also found to be a sufficient substrate of Lys-C and lysyl endopeptidase. The inherent advantage of this method is that the phosphorylation site will always be present on the C-terminal residue of the peptide resulting in a distinguishable $y_1$ ion. Fig. 2 shows an electrospray tandem mass spectrum taken on a QSTAR quadrupole orthogonal TOF mass spectrometer of the peptide RELEELNVPGEIVEK* derived from the protein β-casein (residues 1–15) and modified by the above methodology. The identification of aminoethylcysteine (K*) following trypsin/Lys-C digestion and MS/MS facilitates the determination of Ser 15 of β-casein as the phosphorylation site. The aminoethylcysteine residue produces a unique $y_1$ fragment ion with a mass of 165 Da that can be used as a positive marker of a phosphoserine residue, as it does not overlap with the fragment ions of other natural amino acids. This method has also been adapted for solid-phase purification of phosphopeptides from a mixture of peptides with some success. A potential problem could be in the fact that the β-elimination/Michael addition creates $R$ and $S$ enantiomers of the aminoethylcysteine derivative and only the $R$-enantio-mer is efficiently hydrolyzed by trypsin. Nevertheless, this work is the first example of using chemical replacement methods for phosphospecific proteolysis.

3. Concluding remarks

In summary, protein phosphorylation is an important signaling mechanism that is involved in various cellular functions that are indispensable for cell growth and differentiation. Although great strides were made previously through classical biochemistry methods, mass spectrometry has become a powerful tool and the method of choice for characterization of phosphorylation sites on proteins. Recently, a surge in chemical, biochemical, and chromatographic techniques have further complimented the arsenal for the analysis of phosphopeptides by mass spectrometry. The determination of phosphorylation and other post-translational modifications on proteins should advance our knowledge concerning how various organisms regulate distinct biological processes.

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