Enhancement of Ionization Efficiency and Selective Enrichment of Phosphorylated Peptides from Complex Protein Mixtures Using a Reversible Poly-Histidine Tag

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To improve the detection of phosphorylated peptides/proteins, a combination of optimized MS-based strategies were used involving chemical derivatization with a polyhistidine-tag (His-tag) and affinity enrichment of the resulting His-tag peptides on a nanoscale Ni^{2+}-IMAC column. The phosphoserine and phosphothreonine peptides were derivatized using a one-pot β-elimination/Michael addition reaction with a reversible His-tag possessing a thiol-containing Cys residue. The His-tag peptides were enriched selectively by Ni^{2+}-IMAC and released using either imidazole or cleavage with Factor Xa. This novel capture and enzyme-mediated release provided an additional element of selectivity and yielded phosphopeptide-specific modifications with enhanced MS ionization characteristics. The eluted peptides were mapped using MALDI-TOF MS and QTRAP ESI-MS/MS techniques. The results obtained for a model peptide and two tryptic protein digests show that the method is highly specific and allows selective enrichment of phosphorylated peptides at low concentrations of femtomoles per microliter. (J Am Soc Mass Spectrom 2007, 18, 1007–1017)

One of the best-known mechanisms for covalent modification, occurring in as many as one third of eukaryotic gene products, is protein phosphorylation, which regulates fundamental cellular events: cell division, cell growth, and cell differentiation. Because protein phosphorylation and other post-translational modification cannot be predicted using gene sequence, several strategies have been used. The conventional approach for the detection of phosphorylation sites typically involves radioactive labeling of the phosphorylation site in growth media, followed by the isolation of ^{32}P-labeled phosphorylated protein by immunoprecipitation or SDS-PAGE, enzymatic digestion, and Edman degradation [1, 2]. This approach, besides being a potential hazard, suffers from reduced sensitivity arising from the low abundance of phosphoprotein, limited amount of biological sample, inefficient incorporation of the ^{32}P label, and relatively poor sensitivity of automated Edman sequencing.

Mass spectrometry, which has long been used to identify proteins, is now the preferred technique for mapping post-translational modifications in proteins [3–6]. The analysis is rapid, sensitive, and does not require radiolabeling. Two soft ionization techniques—matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS)—have been successful in detecting the phosphorylation sites in proteins [7, 8]. However, in routine phosphopeptide mapping using MS, the following challenges appear. First, negative charges on phosphate groups result in suppression effects or a low efficiency of ionization. Second, the low stoichiometry and abundance of phosphorylated peptides in a mixture of peptides makes the exclusive characterization of phosphopeptides difficult. Third, the hydrophilic character of phosphopeptides may cause them to elute in the void volume in RP-HPLC. Finally, full sequence coverage by specific proteolysis of large phosphoproteins is difficult. These factors underscore the need for continued development of methods of enrichment before mass analysis [5, 8].

A common technique for enrichment of phosphorylated peptides and proteins from a complex mixture is immobilized metal ion affinity chromatography (IMAC) [9]. Phosphoproteins and peptides bind with specificity to immobilized metal ions, such as Fe^{3+}, Al^{3+}, and Ga^{3+} and usually elute at high pH values. However, the elution of phosphopeptides from IMAC resins can result in the loss of strongly bound phosphopeptides. Also, non-specific binding of negatively charged species may occur during the IMAC procedure [8–10]. In this case, a methylation step is essential to partially reduce the non-specific binding of highly negatively charged peptides to the resins [11]. Alkaline

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phosphatase treatment of the IMAC-enriched peptide, which causes the loss of a phosphate group and shifts the mass by −80 Da, can further confirm the existence of phosphorylation sites and also enhances ionization efficiency [12]. The use of strong cation exchange chromatography before IMAC purification to further reduce the complexity of the sample and to yield a better coverage of monophosphorylated peptides has also been reported [13, 14]. Recent studies have shown that nanocomposites such as titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2) have a high loading capacity and specificity for phosphopeptides in large-scale analyses of protein phosphorylation in biological mixtures [15, 16]. Two MS-based strategies—precursor ion scanning of *m/z* 79 in negative-ion mode and neutral loss scanning of *m/z* 98 in positive mode—have been used for selective detection of phosphorylated peptides within a complex mixture [17, 18].

Chemical derivatization strategies based on β-elimination of the phosphate moiety of phosphopeptides and Michael addition reaction with S- and N-nucleophiles, such as ethanethiol, ethanedithiol, and ethylenediamine, are increasingly being described [19, 20]. In a recent study, Knight et al. [21] applied a phosphorylation-specific proteolysis technique, which converts the phosphorylation sites into lysine analogs using a β-elimination/Michael addition reaction. The resulting modified peptide can then be cleaved phosphospecifically by LysC endoprotease. Other chemical derivatization approaches have been developed based on the incorporation of biotin or fluorous affinity tags to aid the enrichment of phosphopeptides [22–24]. Although each of these methods has its own advantages and certain limitations, the development of new approaches for unambiguous, specific, and selective analysis of phosphoproteins to reduce suppression effects and enhance the detectability of phosphopeptides is still ongoing.

In the present work, a β-elimination/Michael addition reaction is used to replace the phosphate moiety of phosphoserine and phosphothreonine peptides with a His-tag. The thiol group on the side chain of Cys of the His-tag reacts with the dehydroalanine residue intermediate that is formed by the loss of phosphate moiety in alkaline conditions. His-tag peptides are enriched selectively by Ni^{2+}-IMAC and mapped using MS and MS/MS techniques. Removal of the reversible His-tag ligand is achieved using Factor Xa, which cleaves the His-tag peptide at the C-terminal side of Arg of the recognition sequence, thus liberating the immobilized peptide. Our novel enrichment strategy, in addition to the affinity purification of phosphopeptides from a complex mixture, improves the selectivity and specificity of analysis as a result of the specific recognition site of protease Factor Xa that is engineered into the His-tag linker. It is well known that the detailed analysis of MS/MS spectra of phosphopeptides with multiple phosphorylation sites using ion trap instruments remains challenging because of neutral loss of 98 Da from each site. In our study, the final peptide used for MS analysis incorporates a Gly-Cys dipeptide linked through the thiol side chain of Cys at the site of phosphorylation. This modification significantly improves ionization efficiency because of the substitution of the negatively charged phosphate group, aids peptide fragmentation and thus facilitates identification of the precise sites of phosphorylation.

Experimental

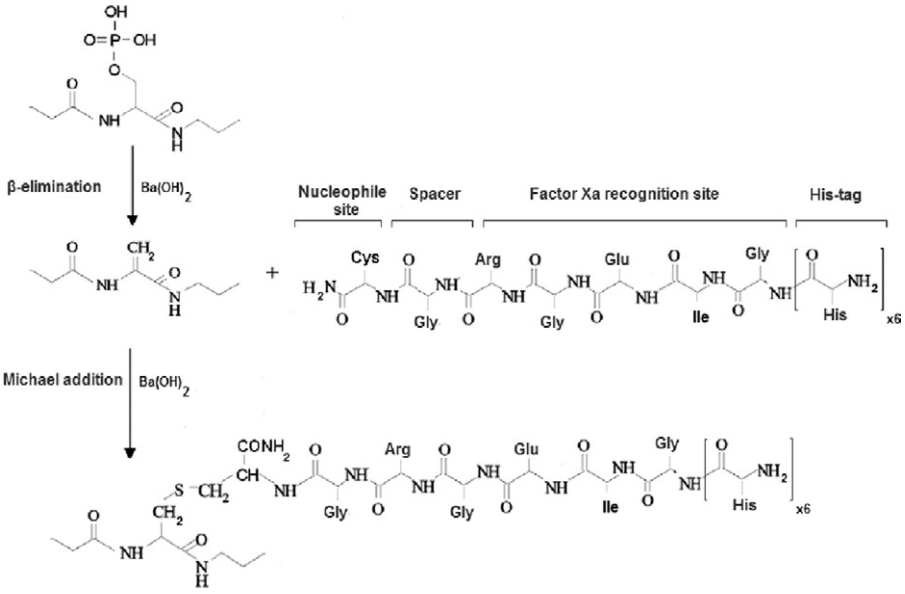
Amino acids and solvents were purchased from Novabiochem (San Diego, CA, USA) or Fisher Scientific (Fair lawn, NJ, USA). α-Casein (bovine) was purchased from Sigma–Aldrich/New Jersey Center for Biomaterials (Piscataway, NJ, USA). Inhibitor-1 (I-1) was provided by Dr. James Bibb (UT Southwestern Medical Center at Dallas, TX, USA). The materials used were barium hydroxide (Alfa Aesar, Ward Hill, MA, USA), gallium nitrate [$Ga(NO_3)_3$], guanidinium HCl, formic acid, trifluoroacetic acid (TFA), hydrogen peroxide (Sigma–Aldrich), Poros 20MC (PE Biosystems, Framingham, MA, USA), Factor Xa (Qiagen, Valencia, CA, USA), titanium oxide (ESPI, Ashland, OR, USA), and sequencing-grade modified trypsin, porcine (Promega, Madison, WI, USA). All matrices, α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and sinapinic acid were purchased from Agilent Technologies (Palo Alto, CA, USA).

Synthesis of His-tag and Model Phosphopeptide

The His-tag peptide (*m/z* 1512.69, HHHHHHGIEGRGC) and model phosphopeptide [*m/z* 1732.75, RPHFPQFS(PO_4)YSASGTA], representing the C-terminal tryptic fragment of RAC-α serine/threonine-protein kinase (P47196) were synthesized on an automated ABI 433 peptide synthesizer (Applied Biosystems, Foster City, CA, USA) using optimized Fmoc/tBu chemistry on Fmoc-amide resin [26]. Crude peptides were purified by RP-HPLC (Waters, Milford, MA, USA) and then analyzed with MALDI-MS to confirm their identity. The purified fractions were pooled together and then lyophilized.

His-tag Derivatization

For the model phosphopeptide; the β-elimination/Michael addition reaction was performed using 10 μL of phosphopeptide (10 pmol), 10 μL nucleophile (1 nmol), 5 μL of 8 M guanidine HCl, 5 μL of ACN, and 10 μL of saturated barium hydroxide. Addition of these derivatizing chemical reagents further diluted the 10 pmol of phosphopeptide to a final concentration of 250 fmol/μL. The reaction was allowed to proceed for 3 h at 45 °C. For α-casein and I-1 protein samples, before derivatization, the protein mixture was subjected to performic acid oxidation by adding 50 μL of 30% aqueous hydrogen peroxide: 95% formic acid (5:95 vol/vol) to 20 μg of protein. The reaction mixture was



Scheme 1. β-Elimination and Michael addition reaction of phosphorylated peptide.

incubated overnight at 4 °C and dried in a centrifugal vacuum system. The dried sample was dissolved in 100 μL of 100 mM ammonium bicarbonate. Trypsin digestion of oxidized proteins was performed at 37 °C overnight using the substrate/enzyme ratio of 100:1. The resulting mixture was diluted to 1 pmol/μL using 0.1% formic acid. The resulting solution (10 μL) was used for derivatization. The derivatization of the resulting tryptic phosphorylated peptides followed the same procedure as described above, giving a final concentration of 250 fmol/μL.

Ga^{3+}-IMAC Enrichment

The method was performed according to the Millipore (Bedford, MA, USA) instructions for ZipTip® (www.millipore.com). Briefly, the tip was equilibrated by washing with 10 μL of freshly prepared 0.1% acetic acid in 50% ACN and primed using 10 μL $Ga(NO_3)_3$ in 10 mM HCl. Then the column was washed first with 10 μL of Milli-Q water, followed by 10 μL of 1% acetic acid containing 10% ACN. The samples were diluted with 10 μL of 0.1% acetic acid, applied to the top of the column, washed with 0.1% acetic acid, followed by 0.1% acetic acid in 10% ACN, and finally rinsed with Milli-Q water. The phosphopeptides were eluted from the column with 10 μL of 0.3 M ammonium hydroxide solution and the eluent acidified before C18 ZipTip desalting and MALDI-MS analysis.

TiO_2 Enrichment

TiO_2 powder was resuspended in 80% ACN in 0.1% TFA and then packed into an Eppendorf gel-loader tip, which was blocked with a glass-wool frit. To optimize the binding of protein to the TiO_2 micro-column, the pH values of the digested proteins were adjusted to 6.5. The resulting mixture was introduced into the TiO_2 micro-column and washed with 0.1% acetic acid followed by 50% ACN in 0.1% acetic acid. The bound phosphopeptides were eluted with 0.5% ammonium hydroxide (pH 9.5).

$His\text{-}tag/Ni^{2+}$-IMAC Enrichment

The His-tag peptides were enriched using nanoscale Ni^{2+}-IMAC. First, the Poros 20MC was prepared by suspending the resin in 20 mM $NiCl_2$ in 10 mM HCl. A nanoliter IMAC column was prepared by loading the IMAC resin into an Eppendorf gel-loader tip. The resin was washed with deionized water and the binding buffer (20 mM Tris HCl, 5 mM imidazole, 0.5 M NaCl, pH 8). A 5μL sample was diluted further with 5 μL binding buffer and loaded on the top of the column. The bound His-tagged peptides were washed with a buffer containing 60 mM Tris HCl, 5 mM imidazole, 0.5 M NaCl (pH 8), and either eluted with solution containing 20 mM Tris HCl, 0.5 M imidazole, 0.5 M NaCl (pH 8) or treated with 10 μL Factor Xa to liberate the immobilized peptide. (Note: His-tag/Factor Xa recognition site, HHHHHHGIEGR, remains attached to the IMAC.) The samples were desalted using Millipore C18 ZipTip (Millipore), washed with 10 μL of 1% formic acid, and finally eluted with 50% ACN containing 1% formic acid. The eluent was analyzed on MALDI-MS and QTRAP ESI-MS/MS.

Mass Spectrometry Analysis

Mass spectrometry analyses were performed using a time-of-flight MALDI-MS (Micromass LR MALDI-TOF, Manchester, UK) and a 4000 QTRAP triple quadrupole/

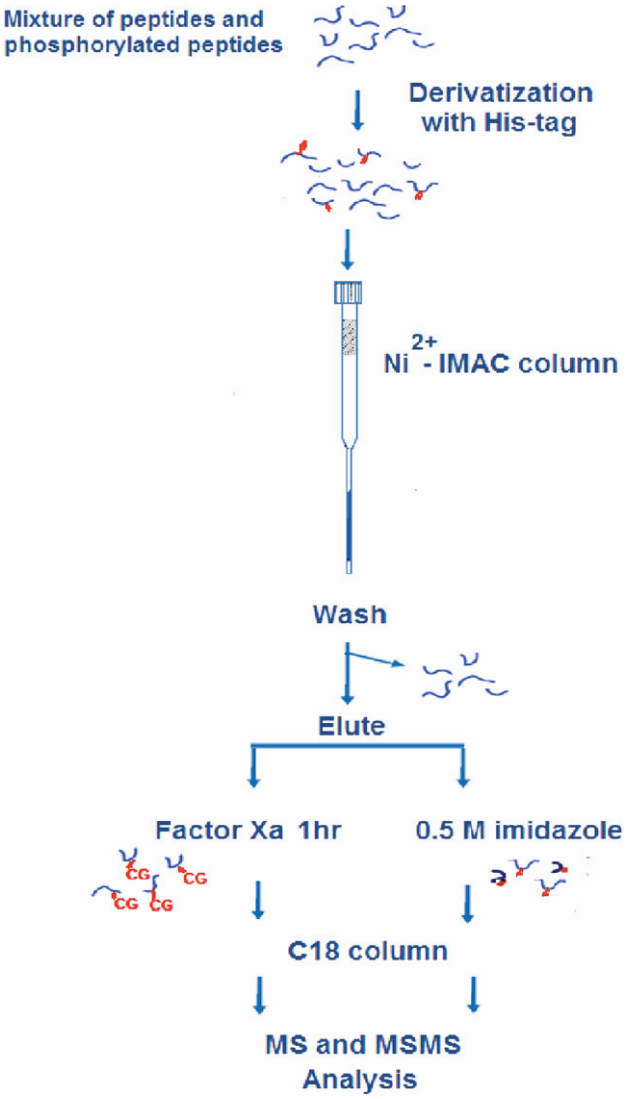
linear ion trap (MDS Sciex, Ontario, Canada). The following settings were used for the QTRAP experiments: nano-ionspray voltage +2000 V, no heating of the source, curtain gas 20 psi, and declustering potential +110 V. Identification of phosphorylation sites and fragmentation was achieved by using MS product of Protein Prospector database. For MALDI-MS analysis, a 0.5μL aliquot of the sample was deposited onto the MALDI plate and then 0.5 μL of the working matrix (DHB and sinapinic acid) solution was applied to the sample droplet. DHB was used for the lower mass scale 500 to 2000 Da and sinapinic acid was used for identification of His-tag and phosphopeptides for mass range above 2000 Da. The sample-matrix droplet was allowed to air-dry at room temperature. Typically, spectra were obtained using a pulsed nitrogen UV laser (337 nm; 3 ns) in positive linear and reflectron mode; pulse voltage of 2800 mV; source voltage of 15,000 mV, using the MassLynx 4.0 Software (Waters Laboratory Informatics).

Results and Discussion

Analytical Strategy

Chemical modification—based on the alkali-mediated β-elimination of phosphoric acid from phosphoserine and phosphothreonine residues followed by Michael addition—was used for the enrichment and identification of phosphorylation sites. Several bases including barium hydroxide, sodium hydroxide, and ammonium bicarbonate based on previous studies were used for the β-elimination reaction. Although all of these bases generated β-elimination products, the results confirmed that barium hydroxide in ACN gave a higher yield for the attachment of the His-tag derivative to the double bond of the dehydroalanine intermediate. Many reports show that the thiol group is an efficient and fast-reacting nucleophile for Michael addition reactions [19–22]. Therefore, we designed a His-tag sequence consisting of three functional domains separated by Gly spacers. These were six histidines for binding to Ni^{2+}-IMAC, a Factor Xa recognition site (Ile-Glu-Gly-Arg), and a sulfhydryl-containing Cys residue at the C-terminus of the peptide to act as nucleophile for attachment at the site of phosphorylation (Scheme **1**).

Typically, the His-tag is introduced on the N- or C-termini and used as a means to affinity purify recombinant proteins from a bacterial expression system. In our research, we have adopted this approach for enrichment of phosphopeptides using Ni^{2+}-IMAC, which binds the His-tag with micromolar affinity. The bound His-tag peptides were washed and eluted with 0.5 M imidazole. Alternatively, the presence of the Factor Xa recognition site in the His-tag enables the bound peptides to be specifically detached from the column using Factor Xa protease. This protease recognizes the Ile-Glu-Gly-Arg sequence, cleaving the His-tag peptide and liberating the modified sequence peptide



Scheme 2. His-tag enrichment strategy for the selective analysis of phosphopeptides.

with the Gly-Cys dipeptide attached at the site of phosphorylation. The His-tag/Factor Xa recognition site sequence remains attached to the IMAC resin (Scheme **2**).

The specificity of Factor Xa adds a further dimension of selectivity to the analysis by leaving non-specific binders attached to the column. Furthermore, enrichment techniques that rely on binding of phosphopeptides to Ga^{3+} or Fe^{3+}-IMAC often suffer from incomplete elution from the column using high pH buffers. Recently, a mixture of DHB and phosphoric acid has been shown to be an efficient eluent for Fe^{3+}-IMAC and TiO_2-columns [27, 29]. Although this mixture is suitable for MALDI-MS analysis, it is not compatible with ESI-MS, commonly used for phosphopeptide analysis. Factor Xa treatment is potentially a more reliable approach that may ultimately result in better recovery and thus sensitivity. The strategy was tested using the model peptide as well as phosphoprotein α-casein.

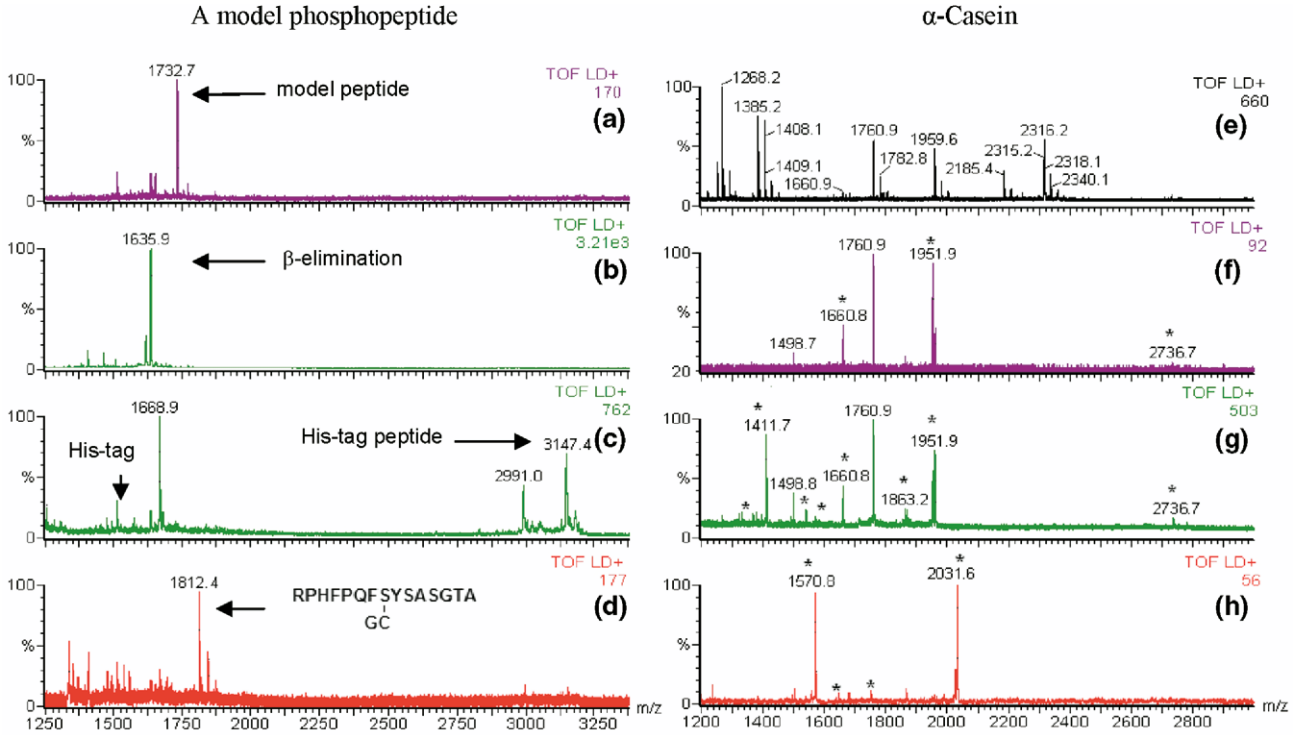


Figure 1. MALDI-MS spectra of (**a**) a model phosphopeptide, (**b**) after β-elimination, (**c**) after derivatization with His-tag (0.5 M imidazole treatment), and (**d**) His-tag peptide after IMAC purification (after Factor Xa treatment). MALDI-MS spectra of α-casein (**e**) before derivatization and after (**f**) Ga^{3+}-IMAC purification, (**g**) enrichment using TiO_2, and (**h**) His-tag derivatization and IMAC purification after Factor Xa treatment. Phosphopeptides and modified peptides marked with *.

Finally, we applied the methodology to investigate the phosphorylation status of I-1 (a substrate for cyclin-dependent kinase 5 and potent inhibitor of protein phosphatase 1). The experiments were performed at least three times to optimize the methodology and test the reproducibility. Qualitatively, the same modified peptides were recovered with each experiment under identical conditions and with similar yield.

Phosphopeptide Enrichment of a Model Peptide

To optimize the reaction conditions for derivatization, a model phosphopeptide (Figure 1a; *m/z* 1732.79) and reversible His-tag, as nucleophile, were used. The MALDI-MS spectrum of the modified peptide shows the peak of *m/z* 1635.92 (Figure 1b) for the dihydroalanine intermediate, resulting from the loss of phosphate. The peak at *m/z* 3147.43 (Figure 1c) corresponds to the His-tag modified peptide. The His-tag peptide was enriched on the Ni^{2+}-IMAC column and bound peptides were eluted with 0.5 M imidazole. The eluent from IMAC was acidified with 5% acetic acid, desalted on a C18 ZipTip, and analyzed by MALDI and QTRAP-MS. All MALDI-MS data were obtained using linear ion mode because it improved and enhanced the signal generated by the phosphopeptides and His-tag derivatized peptides. Holland et al. [28] reported that peaks corresponding to post-translational modified peptides such as phosphorylation and glycosylation, which do not appear in the reflectron mode MALDI analysis, may appear in the linear mode. The peak of *m/z* 3147.43 (calculated *m/z* 3147.46) was clear in the spectrum after enrichment. Treatment of His-tag peptides with Factor Xa, which leaves a Gly-Cys dipeptide at the site of phosphorylation, resulted in a shift in molecular weight to a lower mass of *m/z* 1812.39 (calculated *m/z* 1812.04) (Figure 1d). Non-specific binders such as *m/z* 1668.95 remained attached to the IMAC column because these did not possess the Factor Xa recognition site and, as a result, are absent in the MS spectrum (Figure 1d).

The data obtained show highly selective enrichment before MS analysis because of the combination of nanoscale IMAC, Factor Xa treatment, and the exclusive elimination of interference from other highly acidic peptides. However, MS/MS sequencing is required to unambiguously determine the phosphorylation sites. Figure 2a shows the MS spectrum of +3 and +4 charged ions of His-tag peptide at *m/z* 1050.31 and 788.16, respectively. The peak of the His-tag ligand and its dimer are also present in this spectrum. After purification and treatment with Factor Xa, the His-tag–modified peptide is cleaved at the carboxyl side of Arg, leaving Gly-Cys attached to the tryptic peptide. Consequently, the mass of the doubly charged ion shifts to *m/z* 906.96 (Figure 2b). The model peptide contains a phosphate group on Ser8 and the MS/MS data clearly

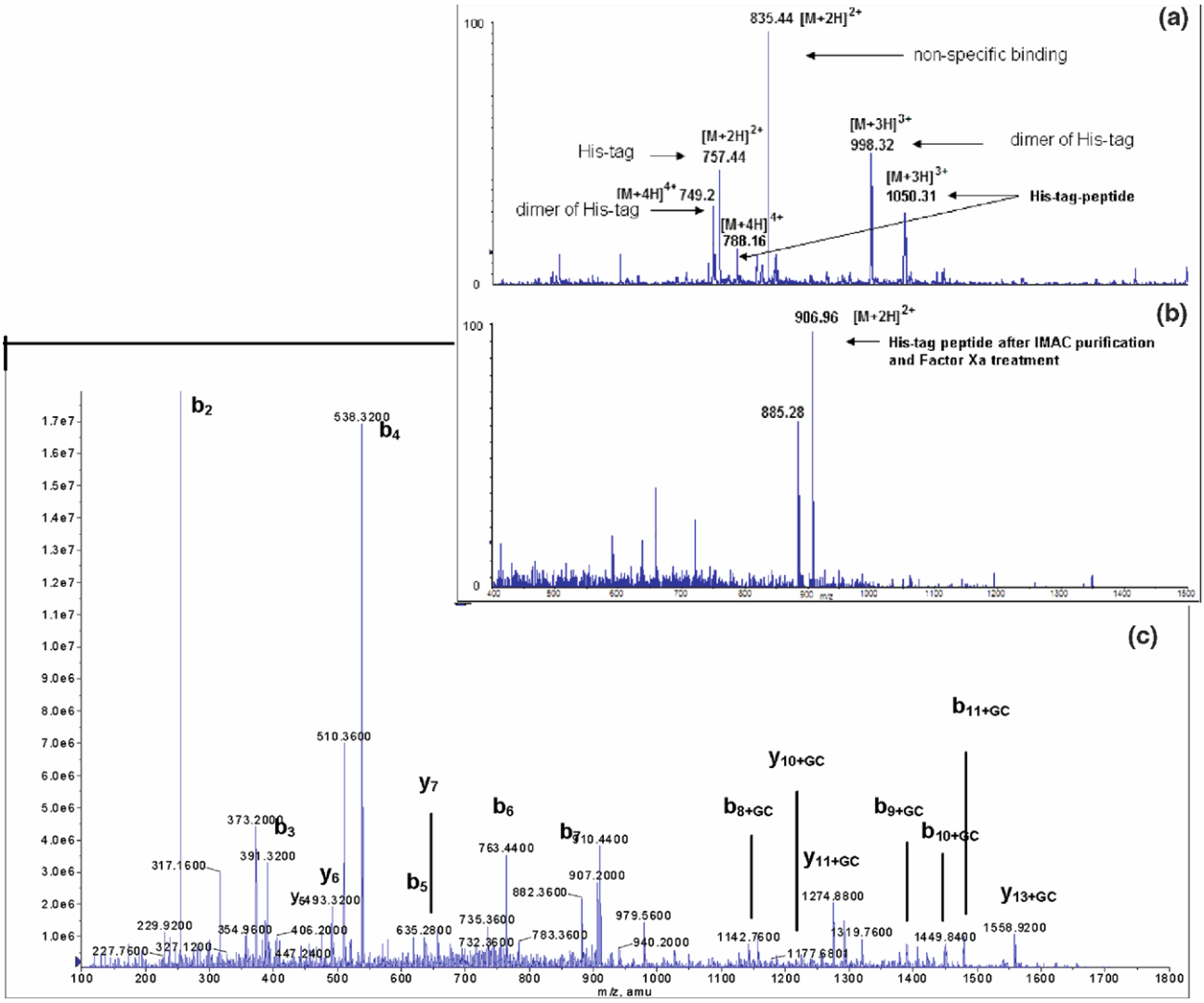


Figure 2. ESI-MS spectrum of (**a**) His-tag peptide before IMAC purification *m/z* 1050.33 for the $[M+3H]^{3+}$ ion. (**b**) ESI-MS and (**c**) ESI-MS/MS spectra of Gly-Cys derivatized peptide (*m/z* 906.96; $[M+2H]^{2+}$ ion), after Ni^{2+}-IMAC purification and Factor Xa treatment.

differentiated this site from the other Ser, Thr, and Tyr residues present in the sequence after its modification with Gly-Cys (Figure 2c).

Phosphopeptide Enrichment of α-Casein

To further evaluate this methodology we decided to use the His-tag for the chemical derivatization of α-casein, a milk-derived protein, which possesses multiple phosphorylation sites. A previous study using mass spectrometry shows the presence of two variants with low sequence homology in the commercially available α-casein. Carr et al. [17] detected nine phosphorylation sites using precursor ion scanning on an ESI/MS instrument. However, they were not able to sequence all the phosphopeptides, especially those phosphopeptides with multiple phosphorylation sites attributed to the ionization suppression effects described previously [17]. Larsen et al. [29] detected ten phosphorylated peptides from α-casein after TiO_2 enrichment using MALDI-MS, including monophosphorylated and multiphosphorylated peptides. When samples were analyzed with ESI-MS, there was a bias towards the monophosphorylated species and several multiphosphorylated species, previously detected by MALDI-MS, were not observed. They attribute this finding to a lower amount of multiphosphorylated peptides compared to monophosphorylated peptides.

Phosphopeptides have traditionally been enriched using Fe^{3+}- and Ga^{3+}-IMAC and, more recently, TiO_2 bound to immobilized supports. To compare the selectivity and sensitivity of the His-tag strategy with these well-established methods, the same solution of α-casein was enriched with either Ga^{3+}-IMAC, TiO_2, or His-tag.

The imidodiacetic acid (IDA) and nitrotriacetic acid (NTA) resins commonly were used as stationary phase for metal ions in IMAC columns. Although NTA resin was superior to IDA resin, non-phosphorylated pep-

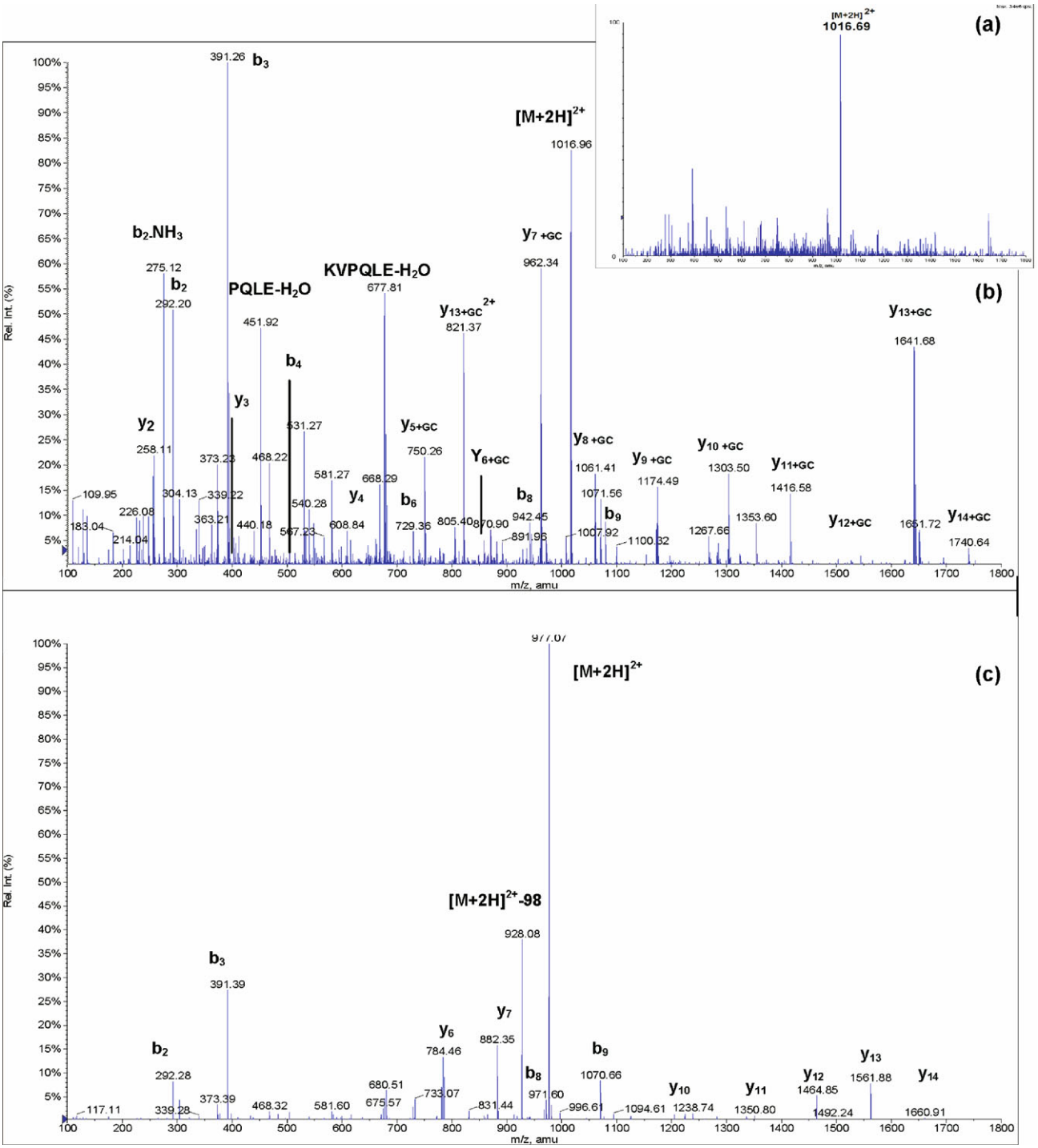


Figure 3. (**a**)°ESI-MS°and°(**b**)°ESI-MS/MS°spectra°of°Gly-Cys°derivatized°α-casein°phosphopeptide α-S1° [119°–134]° after° modification,° IMAC° purification,° and° Factor° Xa° treatment° (*m/z* 1016.54; $[M+2H]^{2+}$° ion).°(**c**)°ESI-MS/MS°spectrum°of°the°precursor°phosphopeptide°YKVPQLEIVPNS*AEER before°His-tag°derivatization°(*m/z* 977.07;°$[M+2H]^{2+}$° ion).

tides,°including°those°containing°multiple°acidic°residues,°are°frequently°enriched°by°this°method.°In°our experiment,°both°IMAC°ZipTip°with°the°IDA°resin°and NTA-Poros°20MC°resins°were°used°for°the°enrichment of°phosphopeptides°and°His-tag°peptides.°Almost°the same°result°was°obtained°with°both°resins.°Three°phosphopeptides°were°identified°using°Ga^{3+}-IMAC°(Figure 1f),°eight°phosphopeptides°from°TiO_2°enrichment°(Figure°1g),°and°four°phosphopeptides°were°readily°detected°using°His-tag°enrichment/Factor°Xa°treatment (Figure°1h).°The°eight°phosphopeptides°identified°using TiO_2°were°α-S2(141–151)°at°*m/z* 1331.53°(one°phosphate

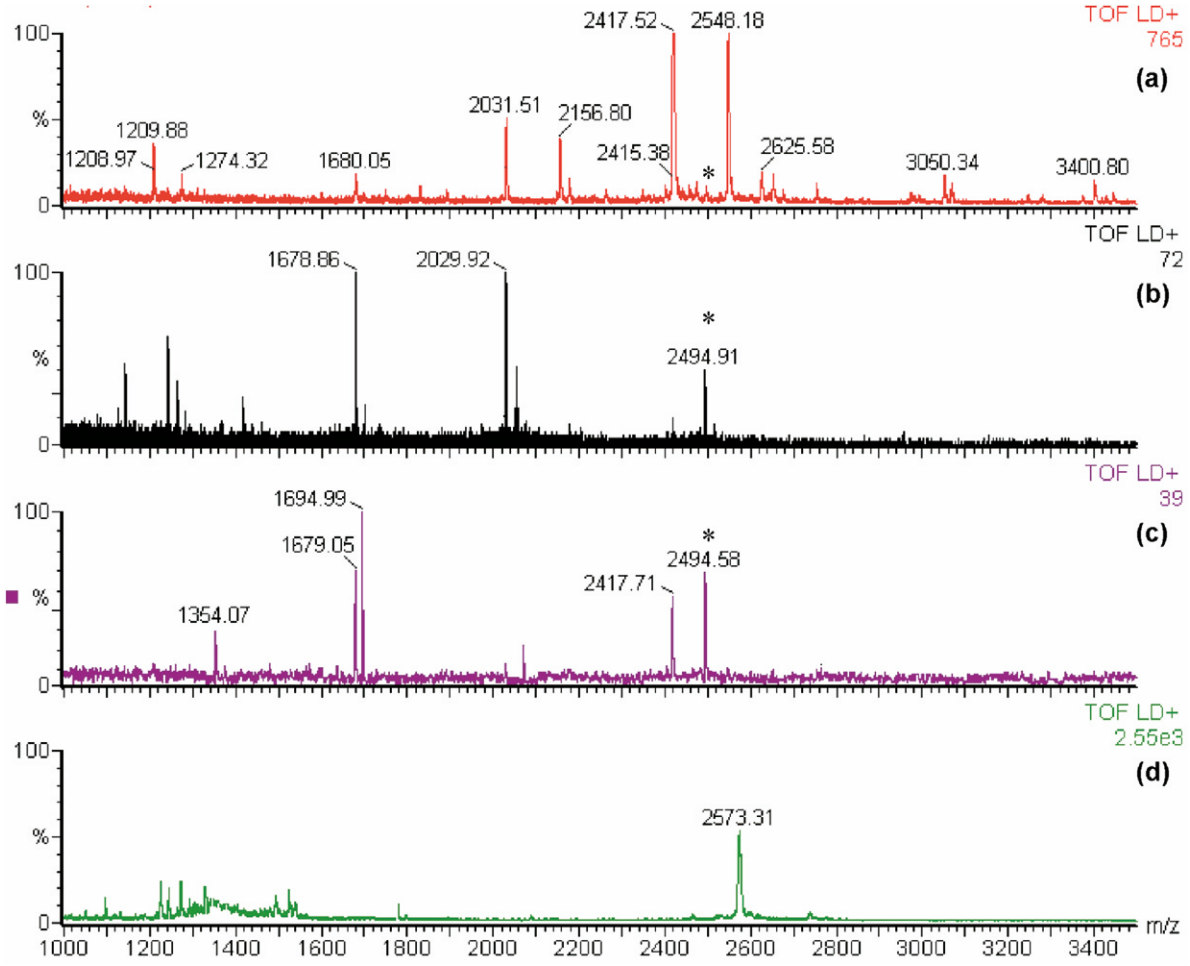


Figure 4. The MALDI-MS spectra of I-1 (**a**) before derivatization, (**b**) after Ga^{3+}-IMAC purification, (**c**) TiO_2 purification, and (**d**) after His-tag derivatization and IMAC purification after Factor Xa treatment.

group) and 1411.71 (two phosphate groups), α-S2(153–165) at *m/z* 1594.65 (one phosphate group), α-S2(141–152) at *m/z* 1539.60 (two phosphate groups), α-S1(121–134) at *m/z* 1660.80 (one phosphate group), α-S1(58–73) at *m/z* 1863.27 (one phosphate group, oxidized form), α-S1(119–134) at *m/z* 1951.95 (one phosphate group), and α-S1(74–94) at *m/z* 2736.73 (five phosphate groups, oxidized form).

In the present work, before chemical derivatization, the proteins were first subjected to performic acid treatment, which oxidizes cysteine, methionine, and tryptophan residues. This pretreatment blocks any undesirable side reactions that may occur between the Cys residue on the His-tag and Cys residues present in the α-casein sequence. The peak of phosphorylated tryptic α-casein peptide at *m/z* 1951.91 (calculated *m/z* 1951.95) gains intensity at *m/z* 3366.66 (calculated *m/z* 3366.67) after His-tag derivatization and IMAC purification (data not shown). The peak of the corresponding purified derivatized peptide after Factor Xa treatment (Figure 1h) appears at 2031.61 (calculated *m/z* 2031.22). Similarly, other phosphopeptides with masses *m/z* 1411.71 (two phosphate groups), 1539.60 (two phosphate groups), and 1660.79 (one phosphate group) shift to *m/z* 1570.85, 1697.51, and 1740.06, respectively, as a result of the substitution of the phosphate group with the Gly-Cys dipeptide (Figure 1h). The replacement of two phosphate groups on serine residues with the Gly-Cys dipeptides in EQLS(GC)TS(GC)EENSK tryptic peptide demonstrates that the method works effectively for multiple phosphorylation sites, including those in close proximity. The MALDI-MS spectrum after enrichment was significantly less complex than that obtained for the tryptic digest of α-casein. Treatment of enriched immobilized His-tag peptides with Factor Xa provided further selectivity and specificity of the method and improved ionization efficiency arising from the substitution of phosphate group with Gly-Cys. It also removed the non-specific binders including the monomeric and dimeric forms of the His-tag ligand that might otherwise interfere with the MS analysis. Compared with the result obtained with TiO_2 column where eight phosphopeptides were observed, the His-tag shows four phosphorylated peptides (two monophos-

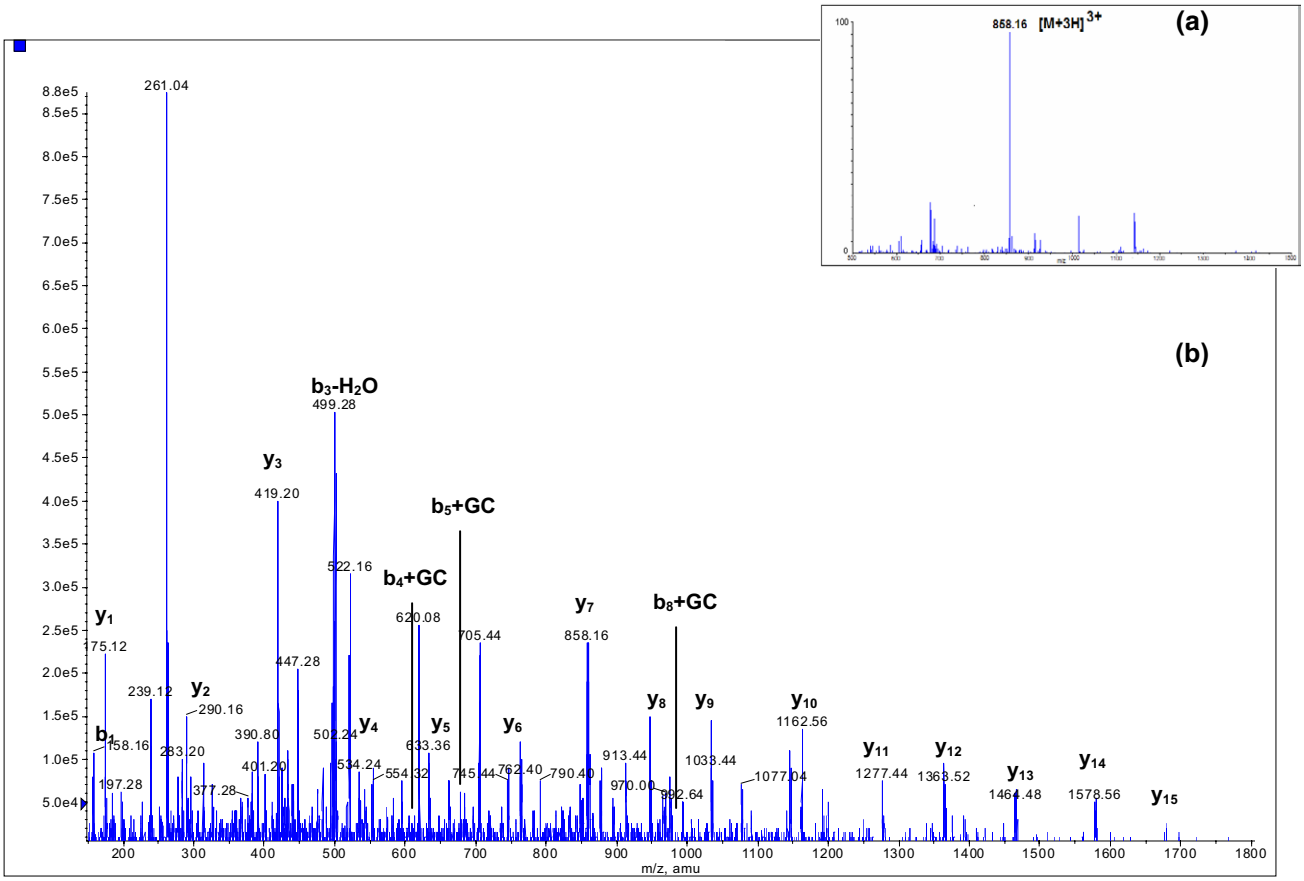


Figure 5. (**a**) ESI-MS and (**b**) ESI-MS/MS spectra of Gly-Cys derivatized I-1 phosphopeptide (*m/z* 858.16; $[M+3H]^{3+}$ ion) after modification, Ni^{2+}-IMAC purification, and Factor Xa treatment. The corresponding sequence is RPT*PATLVLTSDQSSPEVDEDR.

phorylated and two diphosphorylated peptides). The reason could be the low abundance and stoichiometry of the multiphosphorylated species. Data presented by Larsen et al. [29] support this finding as they observed a significantly lower sensitivity for multi-phosphorylated peptides using ESI-MS/MS.

The MS (Figure 3a) and MS/MS (Figure 3b) spectra of the derivatized peptide were obtained using a QTRAP 4000. Although the *y* and *b* ions dominate the spectrum, the series of *y* ions are more complete and better coverage was observed over the high *m/z* region. The fragment obtained corresponds to YKVPQLEIVPNSAEER and the mass of 160.04 Da was added to all fragments from y_{5^+} to y_{15}, which is attributed to the addition of Gly-Cys dipeptide at the site of phosphorylation (Ser12).

To demonstrate that the ionization characteristics of the peptide improved, another MS/MS experiment was performed on the unmodified phosphorylated precursor peptide, using the same collision energy settings (Figure 3c). In the MS/MS spectrum of the corresponding phosphopeptide, the most abundant and intense peak was that of the doubly charged ion highlighting the difficulty of ionizing and fragmenting these phosphorylated species. In comparison, the series of *b* and *y* ions are far more intense in the case of the peptide modified with Gly-Cys. In agreement with the results, substitution of a negatively charged phosphate for a protonated group may improve ionization efficiency of the species of interest, augment CID backbone fragmentation, and, as result, facilitate phosphorylation site identification.

Phosphopeptide Enrichment of I-1

Inhibitory subunit 1 (I-1) is derived from precursor protein phosphatase (PP1), which is a major eukaryotic Ser/Thr phosphatase. PP1 regulates diverse cellular processes such as glycogen metabolism, cell division, muscle contraction, signal transduction, and neuronal functions. PP1 has targeting and inhibitory subunits. The targeting subunits confer substrate specificity and inhibitory subunits suppress the catalytic activity of PP1. I-1 is phosphorylated on Thr35 and Ser67 in vivo. Once phosphorylated on Thr35, I-1 becomes a potent inhibitor of PP1 [30]. The MALDI-MS spectrum of tryptic digest of I-1 before enrichment shows the peak of the phosphopeptide at *m/z* 2494.58 has very low intensity arising from either the low stoichiometry or the low ionization efficiency in the MS (Figure 4a).

In our study, the phosphorylation site on Thr35 of I-1 was investigated using His-tag affinity enrichment and compared to Ga^{3+}-IMAC and TiO_2. Although, the latter techniques are definitely effective for the signal enhancement of low amounts of phosphopeptides from a complex mixture of tryptic peptides, the expected selectivity was not observed. The peaks of acidic peptides were also seen in the spectra but with lower intensity compared to the spectrum before enrichment. For instance, the peaks at *m/z* 1678.92 (ELQTMVEHHLGQQK) and 2030.07, which are the most abundant peaks in the spectrum of I-1, are also evident after enrichment using Ga^{3+}-IMAC and TiO_2 (Figure 4b and c). However, these non-specific sequences are absent in spectra after His-tag enrichment and the peak of Gly-Cys–modified peptide appears with high intensity at *m/z* 2573.31 (calculated mass 2573.82) (Figure 4d). The spectrum after enrichment and elution with Factor Xa is clear from other tryptic peptides and even highly acidic peptides, demonstrating the increased selectivity of the method.

Sequencing the triply charged ion of the Gly-Cys–derivatized peptide at *m/z* 858.52 (Figure 5b) using ESI-MS/MS suggested that the sequence corresponded to RPT*PATLVLTSDQSSPEVDEDR, with Gly-Cys dipeptide providing a convenient marker for identifying the site of phosphorylation.

Conclusion

The combination of chemical derivatization with His-tag and affinity enrichment using Ni^{2+}-IMAC is a highly efficient approach for identification of low-level concentrations of phosphorylated peptides in a complex mixture of peptides. Although the number of identified phosphopeptides is higher using TiO_2, the data presented here show that the treatment of enriched derivatized peptides with Factor Xa improves selectivity by eliminating a greater proportion of contaminating non-specific negatively charged species. Furthermore, substituting a phosphate group with a protonatable Gly-Cys dipeptide offers definite advantages over the phosphorylated precursor when analyzed by ESI-MS/MS in positive mode. In addition, phosphatases, high pH conditions, and ionization in the ion trap MS instruments can cause loss of phosphate groups, resulting in lower sensitivity and increased difficulty identifying the phosphorylation sites. In this respect modification with the Gly-Cys dipeptide facilitates phosphorylation site identification. Results of this research confirm that this innovative method provides a reliable means of phosphopeptide mapping that offers advantages over published enrichment methods.

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