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Facile Identification and Quantitation of Protein Phosphorylation via β-Elimination and Michael Addition with Natural Abundance and Stable Isotope Labeled Thiocholine

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Abstract

Herein, we employ the unique chemical properties of the quaternary amine present in thiocholine (2-mercapto-N,N,N-trimethyl-ethanaminium) in conjunction with alkaline β-elimination and Michael addition (BEMA) reactions for the specific detection, identification and quantitation of phosphorylated serine/threonine containing peptides. Through replacement of the phosphate with thiocholine, the negative charge on the phosphopeptide is switched to a quaternary amine containing a permanent positive charge. This strategy resulted in a 100-fold increase in ionization sensitivity during ESI (sub-500 amol/μL detection limit) accompanied by a markedly enhanced production of informative peptidic fragment ions during CID that dramatically increase sequence coverage. Moreover, the definitive localization of phosphorylated residues is greatly facilitated through the generation of diagnostic triads of fragmentation ions resulting from peptide bond cleavage and further neutral loss of either trimethylamine (−59Da) or thiocholine thiolate (−119Da) during CID in MS^2 and MS^3. Synthesis of stable isotope labeled thiocholine enabled the quantitation of protein phosphorylation with high precision by ratiometric comparisons using heavy and light thiocholine. Collectively, this study demonstrates a sensitive and efficient strategy for mapping of phosphopeptides by BEMA using thiocholine through the production of a diagnostic repertoire of unique fragment ions during LC-MS^2/MS^3 analyses, facilitating phosphosite identification and quantitative phosphoproteomics.

Keywords

Post-Translational Modification; Phosphorylation; Chemical Modification; β-Elimination; Michael Addition; Thiocholine; Mass Spectrometry; Comparative Quantitation

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Introduction

In the human genome, about 2% of the encoded proteins are dedicated to maintaining the appropriate phosphorylation state of targeted proteins through the actions of a wide variety of protein kinases and phosphatases [1-2]. Moreover, approximately 30% of all known proteins in cells are rapidly and reversibly phosphorylated during physiologic and pathophysiologic perturbations. The reversible phosphorylation of cellular proteins is widely believed to be the most important mechanism for the regulation of multiple signal transduction pathways [3-6].

The intrinsic chemical properties of phosphorylated peptides render the detection, identification of phosphosites and quantitation of phosphopeptides a challenging problem in proteomics [7-8]. Peptides containing phosphorylated serine/threonine (pS/pT) residues rapidly undergo neutral loss of phosphoric acid through cyclo-elimination during low-energy CID which leads to the loss of peptide sequence coverage. Moreover, phosphorylated peptides generally have poor ionization efficiencies in the positive ion mode due to the acidity of the phosphate group and ion suppression [9-12]. Complementary approaches have been developed to overcome these difficulties. Enrichment using immobilized metal affinity chromatography (IMAC) after esterification of carboxylates has been useful in many cases [13-15]. However, identification of phosphopeptides is complicated by polydispersity generated by potentially incomplete and nonspecific methyl esterification [16]. Other enrichment strategies, such as use of titanium dioxide [17-18] and zirconium dioxide [19] have shown great efficiency, but these approaches are still hindered by the intrinsic chemical properties of the phosphate moiety that facilitates its neutral loss. The utility of electron transfer dissociation (ETD) which generates c- and z-type ions in which the neutral loss of H_3PO_4 does not occur is becoming increasing appreciated especially for phosphopeptides possessing high charge states [20-21].

Chemical replacement of the phosphate on serine or threonine with other functional groups by β-elimination and subsequent Michael addition (BEMA) was first introduced by Meyers et al. in 1986 [22]. In prior work, Steen et al. demonstrated the efficacy of 2-dimethylamino-ethanethiol followed by hydrogen peroxide oxidation to generate thioester ethanesulfoxide derivatives which produced informative fragment ions during low-energy CID [23]. However, controlled oxidation with the generation of a single predominant reaction product is not straightforward. With cysteamine as the Michael donor, Knight et al. developed a strategy to enzymatically cleave proteins at their phosphorylation sites [24]. Although information on the phosphorylated proteins can be obtained through enzymatic proteolysis, lysines have to be quantitatively blocked through additional chemical reactions to ensure the exclusive cleavage at modification sites. During the development of our strategy, Li et al. used BEMA with several nucleophiles including thiocholine for the detection of phosphopeptides by Raman spectroscopy and mass spectrometry. However, prior mass spectrometric analyses were limited to detection of molecular ions in full MS scans without exploring the unique advantages of this strategy for covalent identification of specific phosphorylation sites through the enhanced generation of informative fragment ions in either the MS^2 or MS^3 modes [25].

In this study we report a novel strategy for the enhanced detection, identification and quantitation of pS/pT containing peptides. Thiocholine is introduced into the peptide at the phosphorylation site via high-yield Ba^{2+} catalyzed β-elimination of phosphate and subsequent Michael addition (Scheme I). Sample complexity has been reduced through reductive alkylation of cysteines and development of optimized BEMA conditions for pS or pT individually. This charge switch strategy exploits the extraordinary sensitivity of the quaternary amine in thiocholine to ionization during ESI resulting in a 100-fold increase in ionization efficiency with detection levels in the sub 500 amol/μL range. The increased endogenous positive charge also engenders higher charge states of thiocholine-labeled tryptic peptides facilitating the production of informative peptidic fragment ions resulting in increased

sequence coverage. Furthermore, phosphopeptide identification has been substantially improved by using the signature neutral losses of trimethylamine (m=59Da) and the thiocholine thiolate (m=119Da) from the thiocholine adduct (Scheme II&III). Thus, CID produces not only the b and y series of peptidic fragment ions but in addition a diagnostic array of fragment ions during MS^2 and MS^3 analyses. Finally, through the synthesis and use of stable isotope labeled thiocholine, quantitative analysis of alterations in the phosphorylation state of proteins during cellular perturbations can be performed through ratiometric comparisons of phosphopeptides containing stable isotope labeled thiocholine to those containing natural abundance thiocholine.

Materials and Methods

Materials

Phosphoprotein β-casein and the phosphopeptide FQpSEEQQQTEDELQDK were obtained from Sigma-Aldrich (St. Louis, MO). The phosphopeptide DHTGFLpTEYVATR was obtained from BIOMOL (Plymouth Meeting, PA); POROS 20 R2 resin was purchased from ABI (Foster City, CA); Slide-A-Lyzer MINI Dialysis Units, 7K MWCO were purchased from Pierce (Rockford, IL); Rapigest was purchased from Waters (Milford, MA); trypsin was purchased from Promega (Madison, MI); α-cyano-4-hydroxycinnamic acid (α-CHCA) solution was obtained from Agilent (Santa Clara, CA). Solvents for mass spectrometric analyses were obtained from Honeywell Burdick&Jackson (Muskegon, MI). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Synthesis of Thiocholine Chloride and Thiocholine-$^{13}C,d_3$ Chloride

Thiocholine chloride was prepared as previously described by Moss et al. [26]. ^1H NMR (D_2O) δ 2.75-2.85 (m, 2H, CH_2S), 3.0 (s, 9H, $N(CH_3)_3$), 3.35-3.45 (m, 2H, CH_2N) (72% yield). S-acetylthiocholine-(N, $^{13}C,d_3$), the precursor for preparing thiocholine-$^{13}C,d_3$ chloride, was synthesized according to Ouyang et al. [27]. Subsequently, thiocholine-$^{13}C,d_3$ chloride was obtained using the same hydrolysis method as that for the naturally occurring isotope described above. ^1H NMR (D_2O) δ 2.75-2.85 (m, 2H, CH_2S), 2.97, 2.98 (d, 6H, $N(CH_3)_2$), 3.35-3.45 (m, 2H, CH_2N) (50% yield).

BEMA of Model Phosphopeptide FQpSEEQQQTEDELQDK with Thiocholine

The phosphopeptide FQpSEEQQQTEDELQDK was modified via BEMA with thiocholine according to the method of Shokat et al. with minor modifications [24]. Lyophilized FQpSEEQQQTEDELQDK was suspended in water to make a 40 pmol/μL stock solution. 12.5 μL of this stock solution was mixed with 9.5 μL DMSO and 3 μL absolute ethanol followed by the addition of 12.5 μL of freshly prepared saturated $Ba(OH)_2$. The reaction was incubated at room temperature under nitrogen for 1 h with gentle vortexing every 20 min. The final pH was 12-13. Next, 25 μL of 1 M thiocholine solution freshly prepared in water was directly added to the reaction. The reaction mixture was incubated at room temperature under nitrogen for 3 h at pH 8-9 and then terminated by the addition of 5 μL of 10% TFA.

BEMA of Model Phosphopeptide DHTGFLpTEYVATR with Thiocholine

Lyophilized DHTGFLpTEYVATR was suspended in water to make a 40 pmol/μL stock solution. 12.5 μL of this peptide stock solution was mixed with 9.5 μL DMSO and 3 μL absolute ethanol followed by the addition of 12.5 μL freshly prepared saturated $Ba(OH)_2$. The reaction was incubated at room temperature under nitrogen for 3 h with gentle vortexing every 20 min. The final pH was 12-13. Next, the β-elimination reaction was terminated by addition of 5 μL of 10% TFA and the resulting solution was desalted with a POROS 20 R2 micro column [28]. The purified peptide solution was dried and reconstituted in 50 μL of 0.5 M thiocholine

solution freshly prepared in 0.1 M NaOH. The mixture was incubated at 50 C° under nitrogen for 5 h at pH 8-9. The reaction was terminated by the addition of 5 μL 10% TFA.

Tryptic Proteolysis of Calcium-independent Phospholipase A_2β Purified from Sf-9 Cells

Hexahistidine tagged iPLA$_2$β was expressed in Sf9 cells and purified as previously described by Jenkins et al. [29]. The resultant enzyme was dialyzed against deionized water for 8 h using a Slide-A-Lyzer® MINI Dialysis Unit, dried in a SpeedVac apparatus (Savant, Holbrook, NY) and reconstituted in 50 μL of 0.2% Rapigest in 50 mM NH_4HCO_3. Next, 2.5 μL of 100 mM DTT was added to a final concentration of 5 mM. The sample was incubated at 60°C for 30 min before 6 μL 150 mM iodoacetamide was added to quench the reduction reaction and initiate alkylation. The sample was incubated for an additional 30 min in the dark. Trypsin was added to the solution at a protease to protein ratio of 1:30 (w:w). The total volume of the sample solution was adjusted to 100 μL with 50 mM NH_4HCO_3. 500 fmol/μL protein sample was incubated at 37 °C for 2 h then acidified with 10 μL 10% TFA to lower the pH to <2. The sample was again incubated at 37 °C for 30 min and centrifuged at 13,000 rpm to pellet the hydrolyzed Rapigest. Half of the resultant supernatant was used for thiocholine modification while the rest of the sample was subject to MS analyses without modification.

BEMA of Trypsinized Calcium-independent Phospholipase A_2β with Thiocholine

Half of the trypsinized iPLA$_2$β sample was dried in a SpeedVac apparatus and reconstituted in 50 μL deionized water followed by the addition of 38 μL DMSO and 12 μL of absolute ethanol. The trypsinized protein solution was then divided into 2 equal aliquots of 50 μL that were separately modified with one of two protocols used to modify pS and pT containing model peptides as described above.

Comparative Quantitation of Phosphoproteins using Natural Abundance Thiocholine and Thiocholine-$^{13}C,d_3$ with the Model Protein β-Casein

50 μL 1 mg/mL β-casein solution prepared in water was mixed with 50 μL of 0.2% Rapigest in 100 mM NH_4CO_3. The protein sample was subjected to in-solution tryptic digestion as described above. The resulting trypsinized β-casein was concentrated with a SpeedVac to ~50 μL and divided to 2 equal aliquots. Aliquot 1 was subject to BEMA with natural abundance thiocholine as the Michael donor while aliquot 2 was modified with stable isotope labeled thiocholine-$^{13}C,d_3$, both using the protocol for FQpSEEQQQTEDELQDK covalent modification as described above. Modified trypsinized β-casein in aliquots 1 and 2 were then mixed in selected ratios (v:v; aliquot 1: aliquot 2, or light: heavy): 5 independent replicates at 1:1 and 3 independent replicates at 1:2, 1:3, 1:4, 4:1, 3:1 and 2:1 ratios respectively were performed. Experimental ratios were compared to expected ratios to evaluate the reproducibility and linearity of the method.

MALDI-TOF/TOF Mass Spectrometric Analyses

MALDI-TOF/TOF mass spectrometric analyses were performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). 1 μL of each peptide sample eluted from a POROS R2 micro column was mixed with 1μL α-CHCA solution and 0.5 μL of the mixture was spotted on an Opti-TOF 384 well plate. Mass spectra of all peptide samples were acquired in the positive ion mode by averaging 500 consecutive laser shots (50 shots per subspectrum with 10 total subspectra) with default calibration. MS^2 analyses of the peptide samples were accomplished by collision-induced dissociation (CID) using air at medium pressure.

HPLC-ESI-MS^2/MS^3 Mass Spectrometric Analyses

Trypsinized protein and peptide samples desalted with POROS 20 R2 micro columns were dried and reconstituted in 0.1% formic acid before injection and separation using a Surveyor HPLC system (Autosampler and Pump, ThermoFisher, San Jose, CA) equipped with a reverse-phase C18 PepMap100 Nano-LC column (75 μm I.D. × 15 cm, 3 μm, 100 angstrom; Dionex, Sunnyvale, CA). Flow rate was maintained at 220-280 nL/min. Samples eluting from the column were directed to the nanospray apparatus (i.e. NanoMate HD with LC coupler, Advion Bioscience Ltd., Ithaca, NY) and sprayed directly into an LTQ-Orbitrap mass spectrometer (ThermoFisher, San Jose, CA) at a spray voltage of 1.7 kV in the positive ion mode. Model peptides and trypsinized β-casein samples were eluted with a gradient from 100% A to 50% A, 50% B in 20 min (Buffer A: 90% water, 10% acetonitrile, 0.1% formic acid; Buffer B: 10% water, 90% acetonitrile, 0.1% formic acid, v:v) and were subject to data dependent MS^2 analyses: full mass scans were acquired using an Orbitrap (300-1600 *m*/*z*, mass resolution=30,000) followed by MS^2 scans in the LTQ of the 5 most intense ions. $iPLA_2\beta$ samples, modified by the two optimized protocols, were analyzed first in survey runs, which consisted of a 90-min gradient from 100% A to 50% A, 50% B and data-dependent MS^2 analyses: full mass scans in the Orbitrap (300-1600 *m*/*z*, mass resolution=30,000) were followed by MS^2 scans in the LTQ of the 5 most intense ions. The ions of interest from the survey runs were then included in the parent mass list of the target runs. The target runs consisted of a 180-min gradient from 100% A to 75% A, 25% B (120 min), then to 50% A, 50% B (60 min) and data-dependent MS^2/MS^3 analyses: full mass scan in the Orbitrap (300-1600 *m*/*z*, mass resolution=30,000) were followed by MS^2 scans in the LTQ of the 3 most intense ions from the parent mass list and the MS^3 scans in the LTQ of the 10 most intense fragment ions following each of the 3 MS^2 scans. The unmodified trypsinized $iPLA_2\beta$ sample was analyzed using the same survey run method as the thiocholine modified samples. The target run for the unmodified sample consisted of a inclusion list generated from the survey run, a 180-min gradient (100% A to 75% A, 25% B (120 min), then to 50% A, 50% B (60 min)), and data-dependent MS^2/MS^3 analyses which consisted of an initial full mass scan in the Orbitrap (300-1600 *m*/*z*, mass resolution=30,000) followed by MS^2 scans of the 5 most intense ions from the parent mass list with neutral loss of H_3PO_4 triggered MS^3 scans in the LTQ. The normalized collision energy for CID was set at 25 for all data-dependent scans.

Data Processing

The local MASCOT server was used to conduct all database searches. A single protein ($iPLA_2\beta$) database was created by in silico trypsinolysis. Thiocholine and thiocholine-$^{13}C,d_3$ with neutral losses of trimethylamine and thiolate were integrated into MASCOT for customized processing of the designed covalent modifications for serine and threonine residues. Carbamidomethyl (C) was set as the fixed modification for trypsinized $iPLA_2\beta$ samples. Common variable modifications included acetylation (N-terminus, K), deamidation (NQ) and oxidation (M). A maximum of two missed cleavages were allowed. Full mass and MS^2 mass accuracy were set at 5 ppm and 1 Da respectively. All identifications by MASCOT were manually verified. All MS^3 scans were analyzed manually.

Results and Discussion

The chemical replacement of the phosphate on serine and threonine residues via β-elimination and Michael addition has been widely used for analyses of phosphopeptides [30-32]. However, an efficient method that concomitantly yields high detection sensitivity, minimal side reactions and informative diagnostic ions suitable for quantitative analyses of both pS and pT containing peptides in the phosphoproteome has remained elusive. Thiocholine was chosen as the Michael donor in this study because of the extraordinary sensitivity of the quaternary amine for ionization during ESI and its utility in creating diagnostic fragment ions resulting from the

neutral loss of trimethylamine and the thiocholine thiolate. Model peptides containing either phosphoserine (FQpSEEQQQTEDELQDK) or phosphothreonine (DHTGFLpTEYVATR) were chosen to optimize reaction conditions for either serine or threonine phosphorylation sites. Phosphorylation of the traditionally employed model protein β-casein and the signaling protein iPLA$_2$β expressed and purified from Sf-9 cells were studied.

Optimization of BEMA Conditions and Ionization Efficiency

The overall reaction yield is an important factor for the success of the BEMA strategy. Since the reaction rates of primary and secondary hydroxyls are quite different for either β-elimination or Michael addition, the reaction conditions were optimized for phosphorylated serines and threonines individually using model phosphopeptides FQpSEEQQQTEDELQDK and DHTGFLpTEYVATR, respectively. Through the use of Ba(OH)$_2$, a controlled high-yield conversion of FQpSEEQQQTEDELQDK to its corresponding dehydro-alanine derivative was accomplished within 40 min; whereas the addition of thiocholine resulted in the rapid (<60 min) synthesis of the desired thiocholine adduct. Based upon integrated UV absorbance, these sequential transformations were accomplished in near quantitative yields (Supplementary Materials). To ensure the completeness of the reaction in more complicated samples, the reaction times were set at 1 h and 3 h for β-elimination and Michael addition respectively.

The mass spectrometric utility of this covalent conversion was demonstrated by a 100-fold increase in ionization efficiency with ESI illustrated by the extract ion chromatogram obtained during reversed-phase chromatography after injection of identical amounts of the thiocholine-modified peptide and its non-modified phosphopeptide precursor. This remarkable increase in ionization efficiency is engendered by the replacement of acidic phosphate with quaternary amine bearing thiocholine, which possesses an endogenous positive charge and is extraordinarily sensitive to ionization during the electrospray process (Figure 1-A). The sensitivity of this method using ESI is at the attomole level (Supplementary Materials). Using MALDI, a 3 fold increase in MS signal was present after derivatization as demonstrated by the analysis of a sample containing equal amounts of phosphopeptide and thiocholine modified phosphopeptide using α-CHCA as matrix (Figure 1-B).

Phosphorylated threonines, possessing secondary hydroxyls, generally have slower reaction rates during both β-elimination and Michael addition in comparison to their primary hydroxyl counterparts [33]. Simply increasing the incubation temperature and/or incubation time results in greater side product formation and is not productive [11,34-35]. However, by purification of the dehydro-alanine intermediate from β-elimination and through the use of nitrogen protection, the overall reaction yield for Michael addition using the model peptide, DHTGFLpTEYVATR, has been greatly improved (Supplementary Materials). Phosphorylated tyrosines are stable and are not altered under the alkaline conditions employed in this study [33].

Fragmentation of Thiocholine-modified Peptides

In addition to dramatically increasing ionization efficiency in ESI-MS, thiocholine derivatization exhibits the unique ability to generate diagnostic triads of informative fragment ions resulting from both routine peptide bond cleavage and facile neutral loss of either trimethylamine (59Da) or thiocholine thiolate (119Da) during CID in MS^2 and MS^3 scanning. This results in a greatly improved identification algorithm for target peptides. A representative ESI-MS^2 spectrum of the triply charged molecular ion of the thiocholine modified peptide FQS*EEQQQTEDELQDK is shown in Figure 2-A. Analysis of the MS^2 fragmentation pattern demonstrated multiple informative b and y ions necessary for sequence identification. As shown in the expanded spectrum, a representative peptidic fragment ion b_5^+ (*m/z*=722.3) is accompanied by its neutral loss counterparts b_5-59^+ (*m/z*=663.2) and b_5-119^+ (*m/z*=603.2) after

loss of trimethylamine or thiocholine thiolate, respectively. The concurrence of these diagnostic triads of fragment ions obtained through conventional CID represent key informative features that can further facilitate the identification of peptides and increase the confidence of assignment of the phosphorylated residue(s) on peptides containing multiple potential phosphorylation sites and those that are multiply phosphorylated. Furthermore, use of MS^3 for the thiocholine-containing ion y_{14}^{+2} (*m/z*=904.4) demonstrated that neutral loss of trimethylamine is the dominant fragmentation pathway leading to a signature neutral loss peak at *m/z* 875.1 (M-59) (Figure 2-B). Thus, introduction of the thiocholine side chain and subsequent fragmentation resulted in the generation of suites of diagnostic triads of fragment ions in both MS^2 and MS^3 that helped to not only enhance sequence coverage, but also increased the confidence of phosphopeptide identification and the specific location of the modified residue. Tandem mass spectrometric analyses of FQS*EEQQQTEDELQDK were also conducted on the singly charged molecular ion in MALDI-MS^2 and the doubly charged molecular ion in ESI-MS^2. In MALDI experiments, the MS^2 spectrum of the molecular ion at *m/z* 2082.62 (+) contains a dominant signature ion peak at *m/z* 2023.7 (+) (Figure 2-C). This peak resulted from the neutral loss of trimethylamine (59Da) from the thiocholine side chain of the molecular ion with minimal sequence-informative b and y ions. The ESI-MS^2 spectrum of the doubly charged molecular ion showed a strong neutral loss peak from the molecular ion at *m/z* 1012.5 (+2) (Supplementary Materials). In MALDI-MS^2, peptides are almost always singly charged [36]. With the low kinetic energy, singly charged thiocholine-containing peptides require higher collision energy to induce peptidic chain fragmentation relative to the neutral loss of trimethylamine. Therefore, the neutral loss is more prone to occur than the formation of b and y ions in low-energy CID, leading to a dominant neutral loss pattern. Similarly, in ESI-MS^2, neutral loss is still a pathway that requires lower energy to induce fragmentation of the doubly charged parent ion. However, when the peptide is triply charged, with the higher vibrational energy derived from the increased kinetic energy gained during acceleration, peptide chain fragmentation becomes more favorable than the neutral loss of trimethylamine leading to the production of a sequence of informative b and y ions [37]. Although neutral loss of trimethylamine is no longer dominant in this case, it can still occur on thiocholine containing fragment ions, which, together with the ions from neutral loss of thiocholine thiolate provides additional confirmatory sequence information and facilitates the assignment of phosphorylation sites. Moreover, as described above (Figure 2-B), neutral loss of trimethylamine becomes favorable again in MS^3, producing a signature neutral loss (SNL) peak during CID which adds an additional dimension to the identification of peptide phosphosites. Although the majority of the tryptic peptides are doubly charged using ESI due to basic residues on both N- and C- termini after conventional trypsinolysis, the modified phosphopeptides typically possess a charge state of 3 or higher due to the additional positive charge introduced by thiocholine. Through combining tryptic proteolysis with thiocholine modification, the majority of the thiocholine modified peptides will be triply charged or higher thereby leading to a rich repertoire of diagnostic triads of peptidic fragment ions and ions from neutral loss of trimethylamine and the thiocholine thiolate for significant improvement in the identification of phosphorylation sites during ESI-MS^2 analyses. In addition, ions containing intact thiocholine side chains losing trimethylamine produce a signature neutral loss pattern in MS^3, greatly improving the confidence of identification of phosphopeptides and the localization of the phosphorylated residue(s) in peptides containing more than one potential phosphosite or in the elucidation of phosphorylation patterns in cases where multiple sites are phosphorylated.

Identification of Endogenous Phosphorylation Sites in Calcium-independent Phospholipase $A_2\beta$ ($iPLA_2\beta$) Expressed in Sf-9 cells

$iPLA_2\beta$ is an important phospholipase in cellular signaling that contributes to diverse cellular processes including arachidonic acid release, insulin secretion, calcium signaling, and

apoptosis [29]. iPLA$_2$β expressed in Sf-9 cells is used to demonstrate the utility of this method in an intact cell system. Prior to BEMA modification, the potential interference of free thiols must be eliminated [39]. Previously, perfomic acid oxidation has been widely used to oxidize cysteine residues and convert disulfide bonds to cysteic acid residues [40-41]. Although treatment with performic acid may convert all cysteines to cysteic acid, it also leads to undesirable oxidation products and an increase in the chemical diversity and complexity of the sample [42-43]. In addition, other potentially susceptible moieties include alkylated cysteines, either endogenous or introduced by reductive alkylation, as well as O-glycosylated serines and threonines. These modified residues could also be converted to the same modified thiocholine residues as pS/pT under alkaline conditions in the presence of strong nucleophiles [44-45]. However, using Ba^{2+} as the catalyst coupled with well-controlled reaction conditions, β-elimination of alkylated cysteines or O-glycosylated serines and threonines occur two orders of magnitude more slowly than β-elimination from pS/pT whereas dephosphorylated serine and threonine residues are unaffected [33]. Under the conditions employed in this study, reductive alkylation does not interfere with BEMA targeting of phosphopeptides and thus many side products and potential ambiguities are avoided. The identification of peptide C^{a}N^{d}VMGPS*GFPIHTAMK in iPLA$_2$β containing both an alkylated cysteine and a thiocholine modified serine residue shows the compatibility of the routine reductive alkylation with Ba^{2+} catalyzed BEMA (Supplementary Materials).

As shown in Table 1, 19 different endogenous phosphorylated sites were identified in iPLA$_2$β using the customized protocols optimized for either pS or pT. These phosphorylation sites originated from a total of 12 unique phosphopeptides. Figure 3-A shows the MS^2 spectrum of triply charged peptide VKEIS*VADYTSHER (24-37). This spectrum consists of b and y ions with diagnostic triads of all thiocholine containing peptidic fragment ions. Shown in the expanded spectrum are representative diagnostic triads from y_{12}^{+2} fragmentation ions resulting from peptide bond cleavage (*m/z*=754.7) and further neutral loss of trimethylamine (−59Da, *m/z*=725.1) and the thiocholine thiolate (−119Da, *m/z*=595.1). The characteristic neutral losses in MS^2 were analyzed by MASCOT to identify the phosphopeptide and the location of the phosphorylation site(s). One of the promising prospects of this approach is the use of these unique and predictable diagnostic triads of fragmentation ions from thiocholine modified peptides in a weighted scoring system for enhanced identification of phosphorylated peptides and phosphosites. Among the ions generated in MS^2 fragmentation, the ten most intense ions were chosen to be further fragmented at the MS^3 level. Examination of the y_{10}^{+2} ion at *m/z* 633.5 (among the top ten ions selected) demonstrated that it also possessed the intact thiocholine side chain, which led to its signature neutral loss pattern in its MS^3 spectrum (Figure 3-B). The ion peak at *m/z* 603.9 corresponds to the neutral loss of trimethylamine from the precursor y_{10}^{+2} at *m/z* 633.5. Examination of the fragmentation pattern of VKEIS*VADYTSHER demonstrated that the thiocholine side chain enabled a unique tandem fragmentation pattern ideal for proteomic analyses, since sequencing of the peptide at the MS^2 level is not comprised by the subsequent neutral losses but rather strengthened by the presence of diagnostic triads of fragment ions in conjunction with the signature neutral loss at MS^3 level.

Conversely, as shown in Table 2, only 6 different endogenous phosphorylated sites, originating from 5 unique phosphopeptides, were identified in iPLA$_2$β samples without thiocholine modification using traditional the MS analysis strategy as described in “Methods”. Figure 4 shows the MS^2 spectrum of the triply charged peptide VKEIpSVADYTSHERVR (24-39), corresponding to the phosphopeptide VKEIS*VADYTSHER (Figure 3-A) with one additional missed cleavage. The spectrum provides minimal sequence informative ions in comparison to that of the thiocholine modified peptide.

Collectively, the present method identifies considerably more phosphorylation sites with higher ion scores and better sequence coverage than the traditional method in the studied cases. The advantages of the current method are to provide important structural information facilitating both the identification of the peptide as well as the location of the precursor ion to facilitate unambiguous identification of the modified residue (i.e., the phosphorylation site(s)). In the current study, 9 out 12 unique sequences demonstrated at least one ion with a signature neutral loss during MS^3 scanning underscoring the utility of the developed method. Although neutral loss information has been incorporated into MASCOT as part of the variable modification of thiocholine, MS^3 spectra with a signature neutral loss pattern must be manually selected at the present time.

Comparative Quantitation of Phosphoproteins using Natural Abundance Thiocholine and Thiocholine-$^{13}C,d_3$ with Model Protein β-Casein

Comparative quantitation via BEMA with thiocholine and thiocholine-$^{13}C,d_3$ was evaluated with the model phosphoprotein β-casein to determine the reproducibility and linearity of the developed method. As described in experimental procedures, two equal aliquots (1 and 2) of trypsinized β-casein were modified with thiocholine and thiocholine-$^{13}C,d_3$ respectively. Modified peptides of β-casein in aliquots 1 and 2 were then mixed at selected ratios (v:v; aliquot 1: aliquot 2, or light: heavy): 5 independent replicates at 1:1 and 3 independent replicates at 1:2, 1:3, 1:4, 4:1, 3:1 and 2:1 respectively. The samples were analyzed by reverse phase HPLC-ESI-Orbitrap as described in "Methods". Tryptic phosphopeptide FQpSEEQQQTEDELQDK was chosen to characterize the quantitation of the phosphorylation of β-casein. The thiocholine and thiocholine-$^{13}C,d_3$ modified peptides FQS*EEQQQTEDELQDK and FQS**EEQQQTEDELQDK were compared by their ion intensities at their prevailing charge state (+3) at the time of elution. The average adjusted experimental ratio of ion intensities for 5 replicates at 1:1 is 0.96 with a standard deviation of 0.02. Thus, this method shows comparable reliability to similar experiments employing isotope-coded affinity tags [46]. The full-mass spectrum from the extract ion chromatogram of two modified peptides at the time of the elution is shown in "Supplementary Materials" with the well recognized "M+4" isotopologue pattern. The experimental ratios of peak intensities were obtained for samples mixed in selected ratios and plotted against theoretical values to yield a correlation coefficient of R^2=0.99 (Supplementary Materials). Furthermore, there was no chromatographic shift between the light and heavy thiocholine modified peptides (Supplementary Materials) and no loss of thiocholine modified peptides during the C_{18} reverse-phase desalting process was observed. Overall, stable isotope ratiometric comparisons demonstrated the anticipated quantitative accuracy in both reproducibility and linearity using heavy and light thiocholine modification.

Conclusion and Perspective

We have demonstrated a strategy for the facile, sensitive and precise detection, identification and quantitation of serine/threonine phosphorylation in proteins. By introducing thiocholine into the target peptides, a powerful mass spectrometric adjuvant is added that greatly increases the sensitivity of phosphosite identification, enriches the repertoire of observable fragmentation ions from the production of a higher charge state, and provides diagnostic triads of fragment ions through signature neutral loss patterns. The development of an affinity purification method for quaternary amines such as those previously demonstrated using calixirenes in conjunction with other complementary ionization and fragmentation methodologies will greatly facilitate the large scale integrated use of this strategy in cellular systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TCH	Thiocholine
DTT	Dithiothreitol
α-CHCA	α-Cyano-4-hydroxycinnamic Acid
HPLC-ESI-MS^2/MS^3	High-Performance Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry
MALDI-TOF/TOF	Matrix-assisted Laser Desorption/Ionization- Tandem Time of Flight
CID	Collision Induced Dissociation
$iPLA_2\beta$	Calcium-independent Phospholipase $A_2\beta$
SNL	Signature Neutral Loss
pS/pT	Phosphorylated Serine/Threonine
LTQ-Orbitrap	Linear Ion Trap in tandem with Orbitrap

References

(1). Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. Science 2002;298:1912–1934. [PubMed: 12471243]

(2). Venter JC, Adams MD, Myers EW. Science 2001;291:1304–1351. [PubMed: 11181995]

(3). Zolnierowicz S, Bollen M. EMBO J 2000;19:483–488. [PubMed: 10675316]

(4). Krebs EG. Philos. Trans. R. Soc. Lond. B. Biol. Sci 1983;302:3–11. [PubMed: 6137005]

(5). Yan JX, Packer NH, Gooley AA, Williams KL. J. Chromatogr., A 1998;808:23–41. [PubMed: 9652109]

(6). Hunter T. Cell 2000;100:113–127. [PubMed: 10647936]

(7). Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. Trends Biotechnol 2002;20:261–268. [PubMed: 12007495]

(8). Steen H, Jebanathirajah JA, Rush J, Morrice N, Kirschner MW. Mol. Cell. Proteomics 2006;5:172–181. [PubMed: 16204703]

(9). Zhou W, Merrick BA, Khaledi MG, Tomer KB. J. Am. Soc. Mass Spectrom 2000;11:273–282. [PubMed: 10757163]

(10). Adamczyk M, Gebler JC, Wu J. Rapid Commun. Mass Spectrom 2001;15:1481–1488. [PubMed: 11507762]

(11). Li W, Boykins RA, Backlund PS, Wang G, Chen HC. Anal. Chem 2002;74:5701–5710. [PubMed: 12463352]

(12). Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Proc. Natl. Acad. Sci. U. S. A 2004;101:9528–9533. [PubMed: 15210983]

(13). Andersson L, Porath J. Anal. Biochem 1986;154:250–254. [PubMed: 3085541]

(14). Posewitz MC, Tempst P. Anal. Chem 1999;71:2883–2892. [PubMed: 10424175]

(15). Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM. Nat. Biotechnol 2002;20:301–305. [PubMed: 11875433]

(16). Mazanek M, Mituloviae G, Herzog F, Stingl C, Hutchins JR, Peters JM, Mechtler K. Nat. Protoc 2007;2:1059–1069. [PubMed: 17545998]

(17). Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. Mol. Cell. Proteomics 2005;4:873–886. [PubMed: 15858219]

(18). Zanivan S, Gnad F, Wickstrom SA, Geiger T, Macek B, Cox J, Fassler R, Mann M. J. Proteome Res 2008;7:5314–5326. [PubMed: 19367708]

(19). Kweon HK, Hakansson K. J. Proteome Res 2008;7:749–755. [PubMed: 18171022]

(20). Chi A, Huttenhower C, Geer LY, Coon JJ, Syka JE, Bai DL, Shabanowitz J, Burke DJ, Troyanskaya OG, Hunt DF. Proc. Natl. Acad. Sci. U. S. A 2007;104:2193–2198. [PubMed: 17287358]

(21). Gunawardena HP, Emory JF, McLuckey SA. Anal. Chem 2006;78:3788–3793. [PubMed: 16737238]

(22). Meyer HE, Hoffmann-Posorske E, Korte H, Heilmeyer LM Jr. FEBS Lett 1986;204:61–66. [PubMed: 3091399]

(23). Steen H, Mann M. J. Am. Soc. Mass Spectrom 2002;13:996–1003. [PubMed: 12216740]

(24). Knight ZA, Schilling B, Row RH, Kenski DM, Gibson BW, Shokat KM. Nat. Biotechnol 2003;21:1047–1054. [PubMed: 12923550]

(25). Li H, Sundararajan N. J. Proteome Res 2007;6:2973–2977. [PubMed: 17583931]

(26). Moss RA, Bizzigotti GO, Huang C. J. Am. Chem. Soc 1980;102:754–762.

(27). Ouyang T, Walt DR. J. Org. Chem 1991;56:3752–3755.

(28). Thingholm TE, Jorgensen TJ, Jensen ON, Larsen MR. Nat. Protoc 2006;1:1929–1935. [PubMed: 17487178]

(29). Jenkins CM, Yan W, Mancuso DJ, Gross RW. J. Biol. Chem 2006;281:15615–15624. [PubMed: 16595686]

(30). Oda Y, Nagasu T, Chait BT. Nat. Biotechnol 2001;19:379–382. [PubMed: 11283599]

(31). Zhou H, Watts JD, Aebersold R. Nat. Biotechnol 2001;19:375–378. [PubMed: 11283598]

(32). Brittain SM, Ficarro SB, Brock A, Peters EC. Nat. Biotechnol 2005;23:463–468. [PubMed: 15768030]

(33). Byford MF. Biochem. J 1991;280(Pt 1):261–265. [PubMed: 1741751]

(34). Li W, Backlund PS, Boykins RA, Wang G, Chen HC. Anal. Biochem 2003;323:94–102. [PubMed: 14622963]

(35). Soderling TR, Walsh K. J. Chromatogr 1982;253:243–251.

(36). Zenobi R, Knochenmuss R. Mass Spectrom. Rev 1998;17:337–366.

(37). de Hoffmann, E.; Stroobant, V. Mass Spectrometry: Principles and Applications. 2 ed. Wiley; Chichester: 2002.

(38). Ma Z, Ramanadham S, Wohltmann M, Bohrer A, Hsu FF, Turk J. J. Biol. Chem 2001;276:13198–13208. [PubMed: 11278673]

(39). Bohak Z. J. Biol. Chem 1964;239:2878–2887. [PubMed: 14216439]

(40). Sanger F. Biochem. J 1949;44:126.

(41). Toennies G, Homiller RP. Anal. Chem 1942;64:3054.

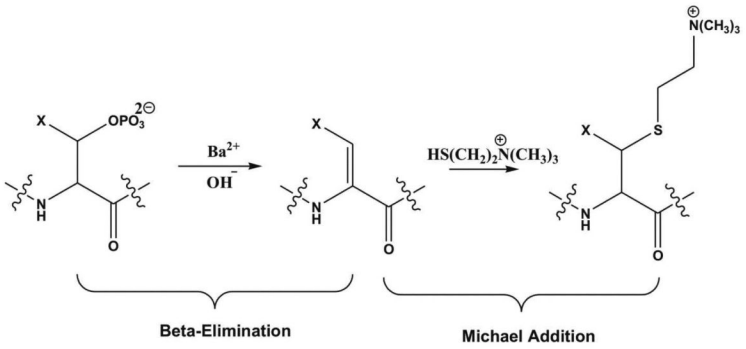
(42). Chowdhury SK, Eshraghi J, Wolfe H, Forde D, Hlavac AG, Johnston D. Anal. Chem 1995;67:390–398. [PubMed: 7856883]

(43). Finley EL, Dillon J, Crouch RK, Schey KL. Protein Sci 1998;7:2391–2397. [PubMed: 9828005]

(44). Wells L, Vosseller K, Cole RN, Cronshaw JM, Matunis MJ, Hart GW. Mol. Cell. Proteomics 2002;1:791–804. [PubMed: 12438562]

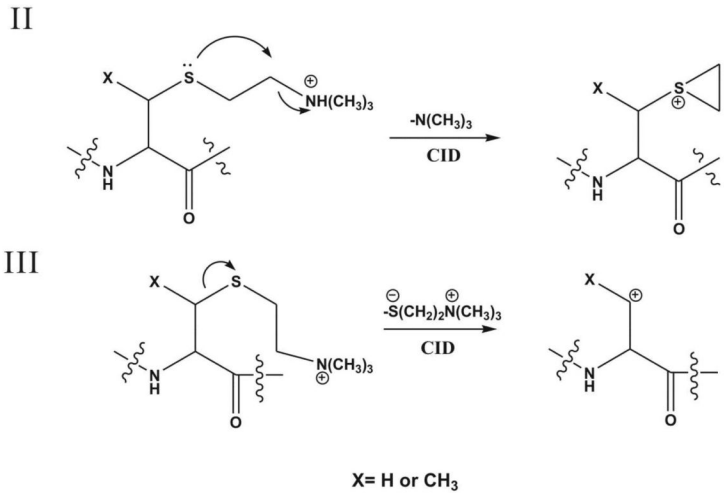
(45). Vosseller K, Hansen KC, Chalkley RJ, Trinidad JC, Wells L, Hart GW, Burlingame AL. Proteomics 2005;5:388–398. [PubMed: 15648052]

(46). Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Nat. Biotechnol 1999;17:994–999. [PubMed: 10504701]



Scheme I.

Chemical modification of phosphoserine and phosphothreonine containing peptides via β-elimination in the presence of $Ba(OH)_2$ and Michael Addition with thiocholine as the Michael donor.



Scheme II/III.

Collision induced dissociation (CID) resulting in the neutral loss of either (II) trimethylamine (m=59 Da) or (III) the thiocholine thiolate (m=119Da) from the thiocholine peptide adduct.

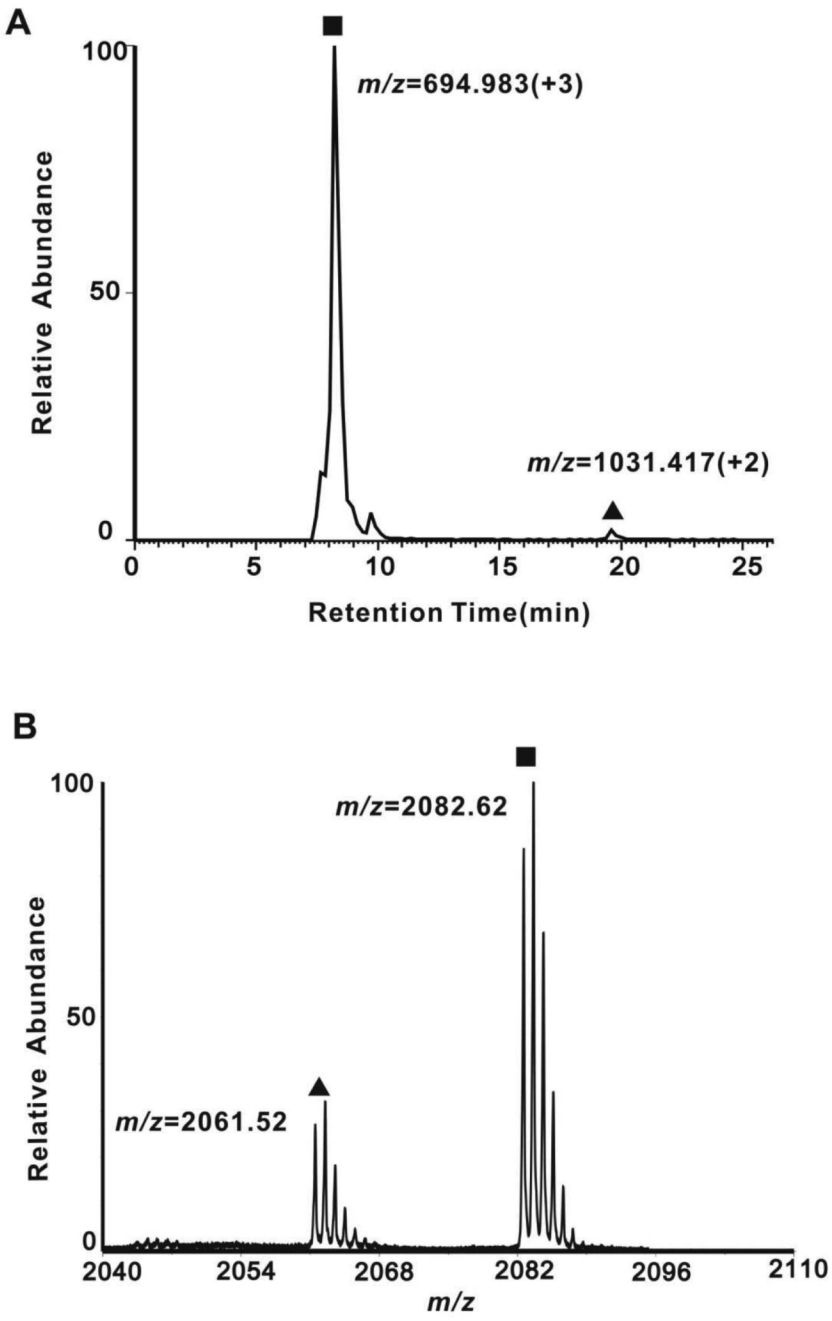


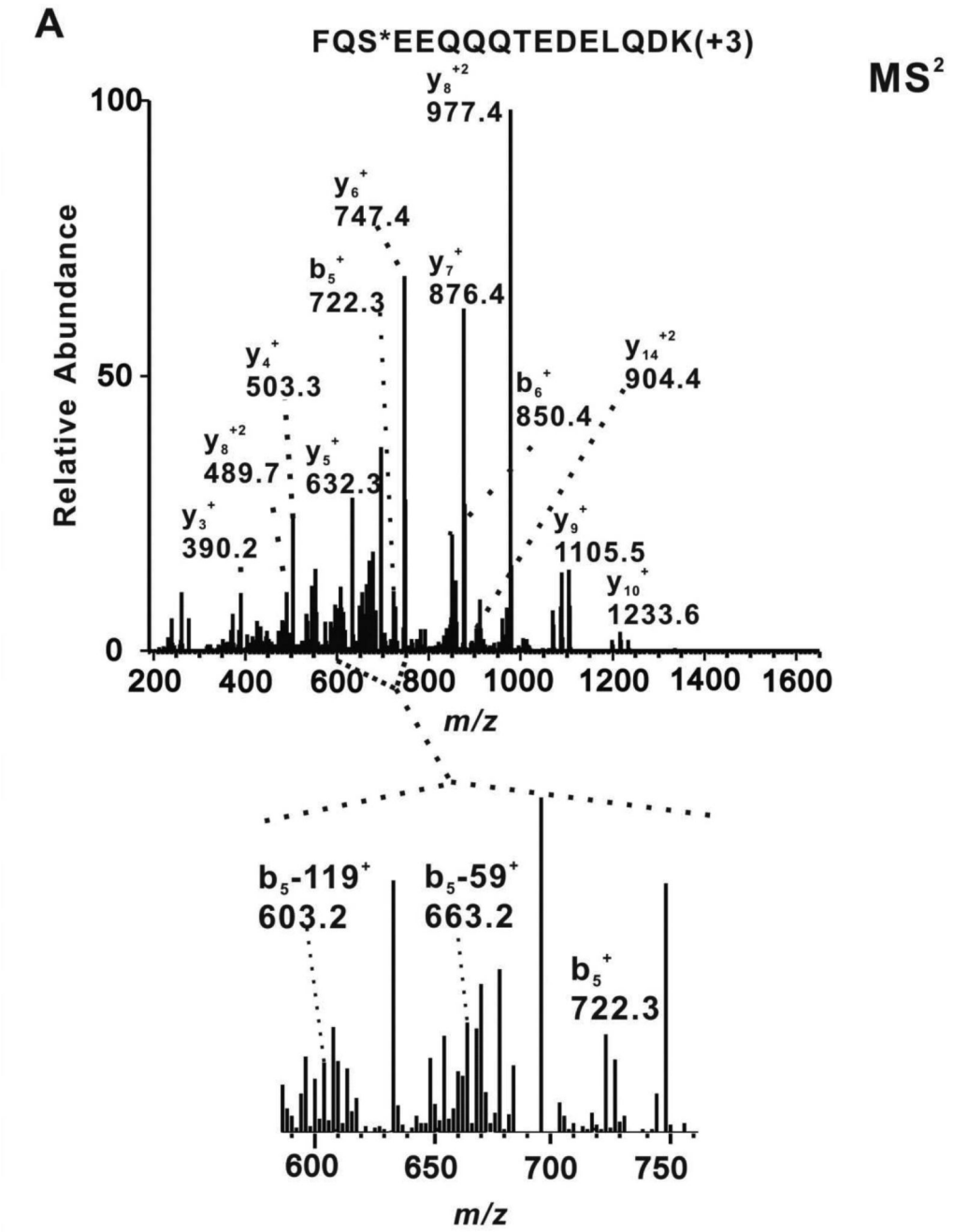
Figure 1. Comparison of the ionization efficiency of the phosphopeptide FQpSEEQQQTEDELQDK (▲) and its thiocholine modified derivative FQS*EEQQQTEDELQDK (■)

The model peptide FQpSEEQQQTEDELQDK was modified via BEMA with thiocholine as described in "Methods" and an equivalent amount of the original unmodified phosphopeptide FQpSEEQQQTEDELQDK was added. The yields of the BEMA reactions were quantitative as shown in "Supplementary Materials". "pS" indicates the phosphorylation site and "S*" indicates the thiocholine modified site.

A. Separation of the peptide mixture using a reverse-phase C_{18} column and analysis employing an ESI-LTQ-Orbitrap as described in "Methods". The full-mass scan extract ion chromatogram

(XIC) of ions at *m/z* 694.983 (+3) and 1031.417 (+2) with normalized ion intensities is presented. The ion at *m/z* 694.983 corresponds to the triply charged molecular ion (dominant charge state) of FQS*EEQQQTEDELQDK (■) and the ion at *m/z* 1031.417 corresponds to the doubly charged molecular ion (dominant charge state) of FQpSEEQQQTEDELQDK (▲). The XIC shows a 100-fold increase in ionization efficiency of the peptide after thiocholine modification.

B. The full-mass spectrum of the 1:1 peptide mixture obtained with a 4800 MALDI-TOF/TOF Analyzer with normalized ion intensity as described in "Methods". The ion peak at *m/z* 2061.52 corresponds to the singly charged molecular ion of FQpSEEQQQTEDELQDK (▲) and the ion peak at *m/z* 2082.62 corresponds to the singly charged molecular ion of FQS*EEQQQTEDELQDK (■). A 3-fold increase in ionization efficiency of the peptide after thiocholine modification was observed with MALDI-MS.



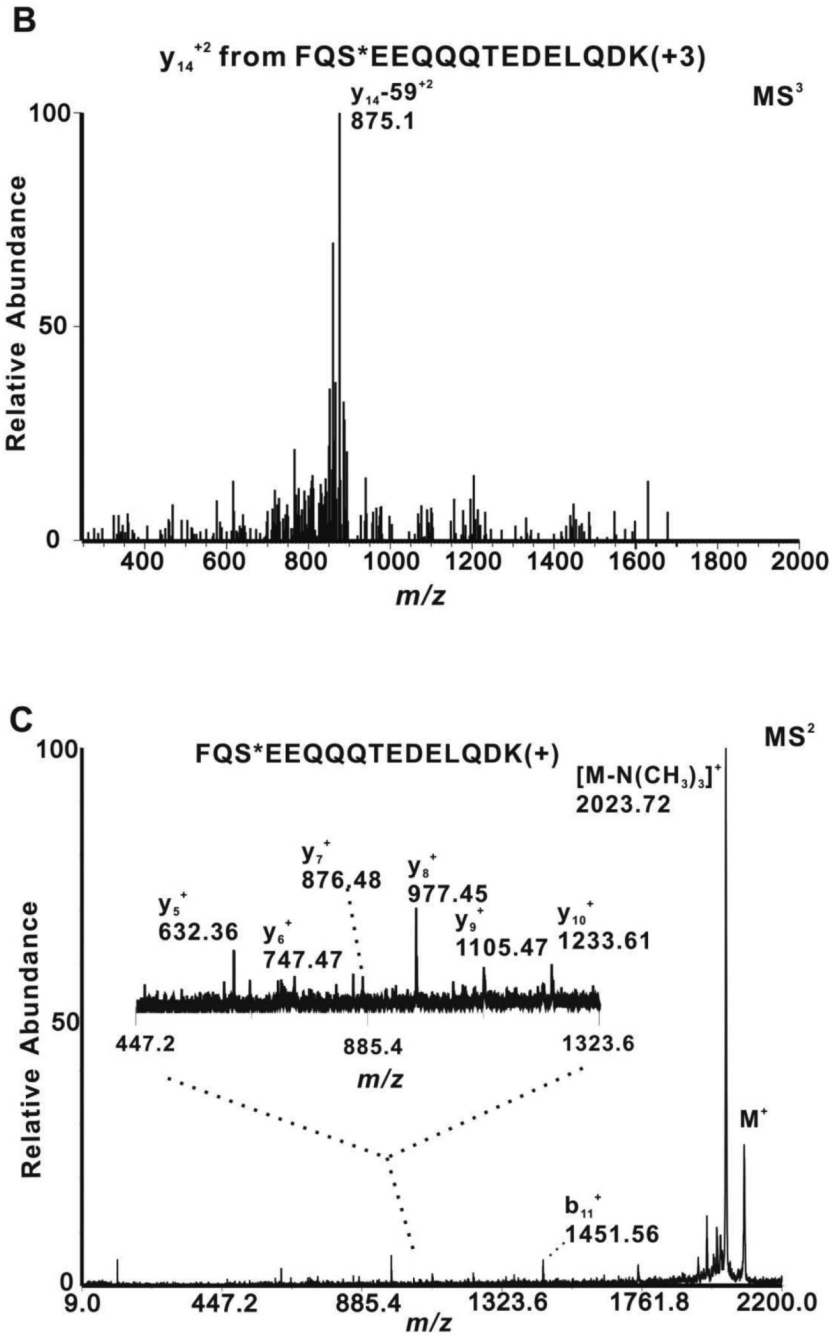


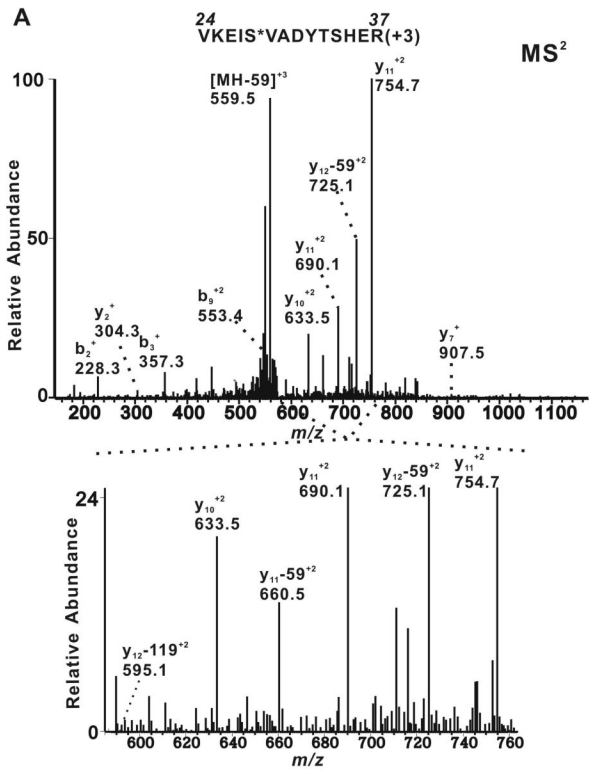
Figure 2. Fragmentation of the thiocholine modified peptide FQS*EEQQQTEDELQDK

A. The MS^2 spectrum of the triply charged molecular ion of FQS*EEQQQTEDELQDK at *m/z* 694.983 was obtained with an ESI-LTQ-Orbitrap as described in "Methods". The fragment ion resulting from the neutral loss of trimethylamine from the parent ion was not detected. Shown in the expanded spectrum is an example of the diagnostic triad consisting of the b_5^+ ion resulting from peptide bond cleavage (*m/z*=722.3) and further neutral loss of trimethylamine (−59Da, *m/z*=663.2) or the thiocholine thiolate (−119Da, *m/z*=603.2). "S*" indicates the thiocholine modified site.

B. The MS^3 spectrum of the y_{14}^{+2} ion at *m/z* 904.4 resulting from the fragmentation of the triply charged molecular ion of FQS*EEQQQTEDELQDK at *m/z* 694.983. The ion peak at

m/z 875.1 corresponds to the doubly charged fragment ion generated from the neutral loss of trimethylamine from the parent ion y_{14}^{+2}.

C. The MS^2 spectrum of the singly charged molecular ion of FQS*EEQQQTEDELQDK at *m/z* 2082.62, obtained with a 4800 MALDI-TOF/TOF Analyzer as described in “Methods”. The ion peak at *m/z* 2023.72 corresponds to the fragment ion resulting from the neutral loss of trimethylamine from the parent ion. Shown in the expanded spectrum are the low-abundance y ions.



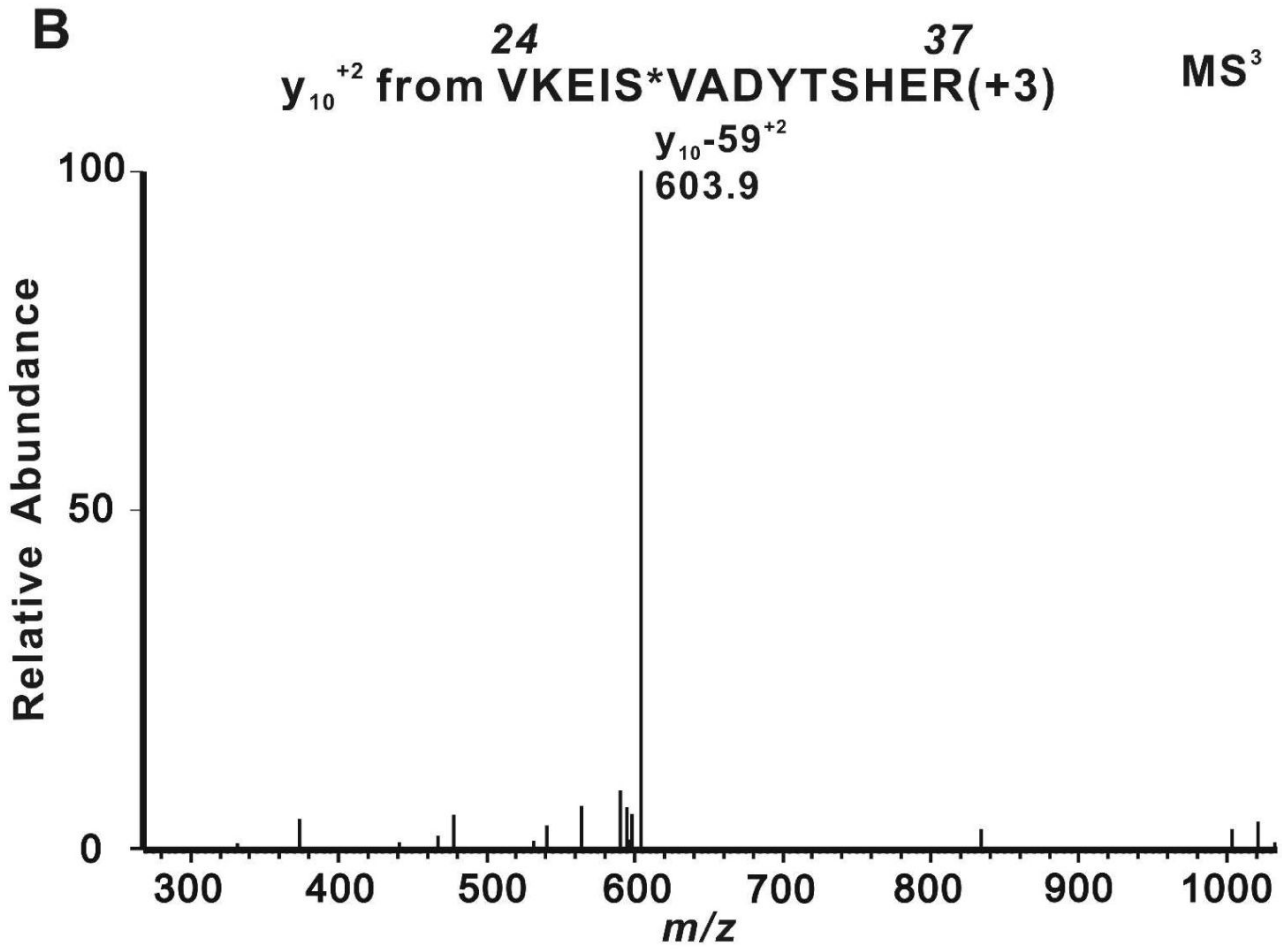


Figure 3. Fragmentation of the thiocholine modified peptide VKEIS*VADYTSHER identified in iPLA$_2$β at both the MS^2 and MS^3 levels

A. The MS^2 spectrum of the triply charged molecular ion at *m/z* 578.966 (VKEIS*VADYTSHER) was obtained with an ESI-LTQ-Orbitrap as described in "Methods". The fragment ion resulting from the neutral loss of trimethylamine from the parent ion was present at *m/z* 559.5. Shown in the expanded spectrum (relative intensity zoom = 24%) are examples of the diagnostic triad consisting of the y_{12}^{+2} fragmentation ions resulting from peptide bond cleavage (*m/z*=754.7) and further neutral loss of trimethylamine (−59Da, *m/z*=725.1) or the thiocholine thiolate (−119Da, *m/z*=595.1). Also shown is the y_{10}^{+2} (*m/z*=690.1) and its corresponding ion peak of the neutral loss of trimethylamine (−59Da) at *m/z*=725.1. "S*" indicates the thiocholine modified site.

B. The MS^3 spectrum of the y_{10}^{+2} ion at *m/z* 633.5 resulting from the fragmentation of the triply charged molecular ion at *m/z* 578.966 (VKEIS*VADYTSHER) obtained with an ESI-LTQ-Orbitrap as described in "Methods". The ion peak at *m/z* 603.9 corresponds to the doubly charged fragment ion generated from the neutral loss of trimethylamine from the parent ion y_{10}^{+2}.

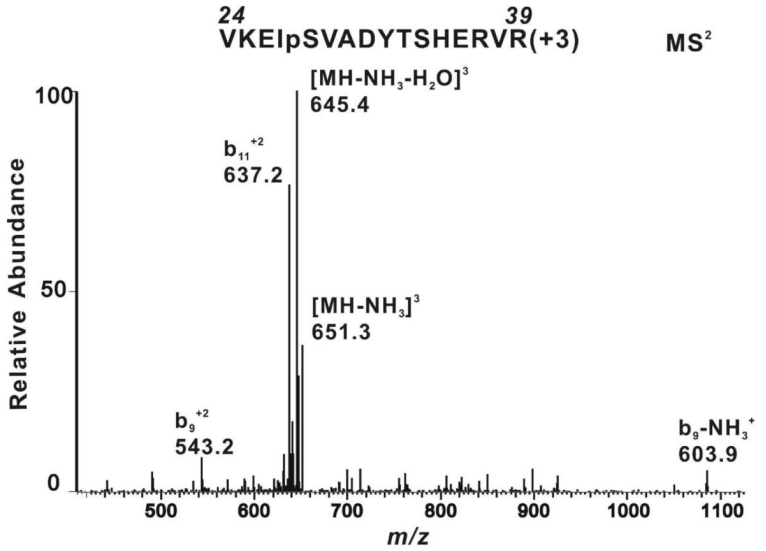


Figure 4. Fragmentation of the phosphopeptide VKEIpSVADYTSHERVR identified in iPLA$_2$β without thiocholine modification

The MS^2 spectrum of the triply charged molecular ion at *m/z* 656.988 (VKEIpSVADYTSHERVR) was obtained with an ESI-LTQ-Orbitrap as described in "Methods". The fragment ion resulting from the neutral loss of H_3PO_4 from the parent ion was not observed. "pS" indicates the phosphorylated site.

Table 1

Identification of endogenous phosphorylation sites in iPLA$_2$β from Sf-9 Cells with thiocholine modification. Tryptic iPLA$_2$β peptides were modified by two different protocols optimized for either phosphoserine or phosphothreonine as described in "Methods". The thiocholine modified peptides were desalted, separated using reverse-phase nanobore HPLC and analyzed with an LTQ-Orbitrap system. Candidate peptides were identified using MASCOT with the designated thiocholine modification as well as common amino acid modifications as described in "Methods"

12 unique phosphopeptides and 19 different phosphorylated residues were identified. "*" denotes phosphorylation sites; "$^{(*)}$" denotes that more than one phosphorylation site was identified in the same peptide. Those that were not concurrent are indicated by "/". The superscripts "^{ace}", "^{ac}", "^{d}" and "^{o}" denote the following modifications: acetylation (N-terminus), acetylation (K), deamidation (NQ) and oxidation (M), respectively. All cysteine residues were carbamidomethylated. Identified peptides with missed cleavages are listed together with the completely trypsinized peptides indicated by bold bordered boxes. Peptides which yielded a signature neutral loss pattern in MS^3 are highlighted in bold.

Sequence#	Sequence	Phosphorylation Site(s)	Ion Score	Δm ppm	Coverage%
24-37	**VKEIS*VADYTS$^{(*)}$HER**	S28/S34	54.62	−2.02	86%
26-37	**EIS*VADYTS$^{(*)}$HER**	S28/S34	34.13	−2.52	92%
38-53	**VREEGQLILFQNAS*NR**	S51	58.41	−1.06	81%
246-261	**CN^{d}VMGPSGFPIHT*AMK**	S252/S258	7.47	−1.03	75%
307-327	GCDVDSTS*AAGNTALHVAVM^{o}R	S315	3.72	−0.98	38%
396-405	**Q^{d}LQDLMPIS*R**	S404	16.69	−3.99	70%
408-417	**KPAFILS*S$^{(*)}$MR**	S414/S415	14.77	−1.36	70%
408-420	**KPAFILS*S$^{(*)}$MRDEK**	S414/S415	19.21	−2.71	69%
479-489	S*MAYMRGVYFR	S479	9.31	−3.31	45%
497-511	GS*RPYES$^{(*)}$GPLEEFLK	S498/S503	33.45	−1.66	80%
513-524	**EFGEHTKMoT*DVK**	T521	9.85	0.72	42%
528-537	**VMLT*GT$^{(*)}$LS$^{(*)}$DR**	T531/T533/S535	33.06	−1.68	90%
582-595	**SS*GAAPT$^{(*)}$YFRPN^{d}GR**	S583/T588	29.26	−1.14	71%
694-705	**AWS*EMVGIQYFR**	S696	34.15	−1.51	92%

Table 2

Identification of endogenous phosphorylation sites in iPLA$_2$β from Sf-9 Cells without thiocholine modification. Tryptic iPLA$_2$β peptides were desalted, separated using reverse-phase nanobore HPLC and analyzed with an LTQ-Orbitrap system. Candidate peptides were identified using MASCOT with the designated thiocholine modification as well as common amino acid modifications as described in "Methods"

5 unique phosphopeptides and 6 different phosphorylated residues were identified. "p" denotes phosphorylation sites; "(p)" denotes that more than one phosphorylation site was identified in the same peptide. Those that were not concurrent are indicated by "/". The superscripts "^{ace}", "^{ac}", "^{d}" and "^{o}" denote the following modifications: acetylation (N-terminus), acetylation (K), deamidation (NQ) and oxidation (M), respectively. All cysteine residues were carbamidomethylated.

Sequence#	Sequence	Phosphorylation Site	Ion Scores	Δm ppm	Coverage%
24-39	VKEIpSVADYTSHERVR	S28	5.7	−2.34	63%
208-218	^{ace}NApSAGLNQVNK	S210	16.3	−2.59	82%
266-282	^{ace}GCAEMIISM^{o}DSpSQIHSK	S277	6.34	1.23	35%
396-407	Q^{d}LQ^{d}DLMPIpSRAR	S404	8.15	−1.86	42%
633-643	LSIVV(p)SLGpTGR	S638/T641	22.87	−3.39	91%