

Detection and Site Localization of Phosphorylcholine-Modified Peptides by NanoLC-ESI-MS/MS Using Precursor Ion Scanning and Multiple Reaction Monitoring Experiments

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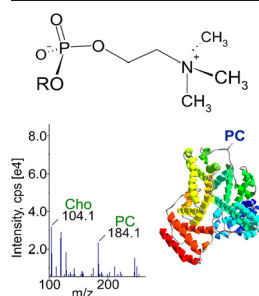
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Abstract. Phosphorylcholine (PC)-modified biomolecules like lipopolysaccharides, glycosphingolipids, and (glyco)proteins are widespread, highly relevant antigens of parasites, since this small hapten shows potent immunomodulatory capacity, which allows the establishment of long-lasting infections of the host. Especially for PC-modified proteins, structural data is rare because of the zwitterionic nature of the PC substituent, resulting in low sensitivities and unusual but characteristic fragmentation patterns. We have developed a targeted mass spectrometric approach using hybrid triple quadrupole/linear ion trap (QTRAP) mass spectrometry coupled to nanoflow chromatography for the sensitive detection of PC-modified peptides from complex proteolytic digests, and the localization of the PC-modification within the peptide

backbone. In a first step, proteolytic digests are screened using precursor ion scanning for the marker ions of choline (m/z 104.1) and phosphorylcholine (m/z 184.1) to establish the presence of PC-modified peptides. Potential PC-modified precursors are then subjected to a second analysis using multiple reaction monitoring (MRM)-triggered product ion spectra for the identification and site localization of the modified peptides. The approach was first established using synthetic PC-modified synthetic peptides and PC-modified model digests. Following the optimization of key parameters, we then successfully applied the method to the detection of PC-peptides in the background of a proteolytic digest of a whole proteome. This methodological invention will greatly facilitate the detection of PC-substituted biomolecules and their structural analysis.

Keywords: Phosphorylcholine, Post-translational modification, ESI-MS/MS, Precursor ion Scanning, Multiple reaction monitoring, Immunomodulation

Received: 26 August 2014/Revised: 17 October 2014/Accepted: 22 October 2014/Published Online: 9 December 2014

Introduction

Phosphorylcholine (PC) has been recognized as a structural component in both prokaryotic and eukaryotic pathogens. First detected in the Gram-positive bacterium *Streptococcus pneumoniae* in 1967, it was later found in Gram-negative bacteria and many important disease-causing parasites, such as protozoa as well as gastrointestinal and filarial nematodes [1,

2]. PC modifications have been observed on teichoic acid, lipoteichoic acid, lipopolysaccharides, glycolipids, and glycoproteins [3–6]. There is first evidence that PC-substituted peptides may occur also in mammals (e.g., on placental secretory polypeptides) [7, 8].

PC-bearing antigens have been found to possess immunomodulatory capacity and to interfere with key proliferative signaling pathways in B- and T-cells as well as in dendritic and mast cells, thus facilitating the survival of parasites within their hosts [1, 9–16]. Therefore, these effects could contribute to the observed low antibody levels and poor lymphocyte responsiveness. Detailed

data on the different types of PC-carrying compounds as well as on their biosynthesis, however, are limited and have only been reported in the last few years [1, 17–19].

For the characterization of the biological function of PC-substituted parasitic antigens, the detailed knowledge of their PC-epitope structures is a prerequisite. So far, mass spectrometric analyses have focused on PC-substituted glycosphingolipids and glycans. PC-conjugated glycosphingolipids have been found in all parasitic and free-living nematodes investigated so far [20–24]. PC-substituted glycosphingolipids show characteristic fragmentation patterns in MALDI-TOF-MS and nano-spray ESI-IT-MS because of the loss of a trimethylamine group by Hoffmann elimination and formation of cyclic phosphodiester [22–26]. Likewise, protein-bound PC-epitopes have been detected in various species of this phylum [20, 27–35]. The mass spectrometric analysis of these PC-substituted biomolecules, however, is often hampered by a significantly lower sensitivity attributable to the zwitterionic character of the PC-substituents, the dominance of fragments derived from the zwitterionic residue, and the absence of structure specific fragment ions [36]. Although PC has been mostly described to be linked to proteins via N-glycans, there is evidence that other types of linkages might also occur as proposed for the aspartyl protease ASP-6 from *Caenorhabditis elegans* [18, 33, 37–41]. Furthermore, PC might also be directly linked to the protein backbone, similar to the modification of type IV pili of *Neisseria gonorrhoea* [32–35, 42]. Additionally, 1-O-alkenyl-sn-glycerol-3-phosphorylcholine modifications bound via an ester-linkage to placental peptides have been proposed [8].

Investigations on chemically synthesized peptides with the PC residue coupled to amino acid side chains by either phosphoramidate- or azophenyl linkages showed characteristic fragmentation reactions similar to PC-substituted glycosphingolipids, leading to cyclic phosphate derivatives, which are highly stable towards further fragmentation, thus hampering a subsequent localization of the PC-unit [43]. However, attempts to detect PC-substituted peptides from crude proteolytic digests have failed so far.

Here we report a targeted mass spectrometric strategy based on precursor ion scanning and multiple reaction monitoring (MRM) that allows for the very first time the detection of PC-substituted peptides resulting from a tryptic digest of PC-modified bovine serum albumin (BSA) and on a background of a tryptic digest from a whole proteome. Furthermore, MRM-triggered MS/MS analyses allowed the identification of the peptide and confident localization of the modification site on the peptide backbone. This development will significantly improve the mass spectrometric methodological repertoire for the structural elucidation of PC-epitopes.

Experimental

Materials

The synthetic peptide PVQNKFFGLA-amide (CS-01) was purchased from GeneCust (Dudelange, Luxembourg). BSA was from Sigma (Deisenhofen, Germany).

Preparation of PC-Peptides and PC-BSA

For the synthesis of PC-phosphoramidate conjugates phosphorylcholine (15 μmol ; calcium chloride salt) was activated by reaction with equimolar amounts of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma) in 0.5 mL water and incubation at room temperature (RT) for 30 min. For derivatization, 1 mg (890 nmol) of the peptide was added to the EDC-activated PC and incubated for 1 h at RT [44].

For the synthesis of the PC-azophenyl conjugates, 5 mg *p*-aminophenyl-PC (TCR, Toronto, Canada) was dissolved in 50 μL 1 M hydrochloric acid containing 1.3 mg sodium nitrite (Merck, Darmstadt, Germany). After 10 min, the reaction mixture was added to 300 μL of peptide solution (500 μg peptide in 0.1 M sodium borate, 0.15 M sodium chloride, pH 9.0) and the reaction was performed under vigorous shaking for 16 h at 4°C [45]. The reaction mixtures were stored at –20°C.

The PC-peptides were separated from educts and side-products by reversed-phase chromatography on a Repronil Pur C18 AQ column (4 mm i.d., 150 mm length, 5 μm particle size; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using a Hewlett Packard HP1090 HPLC system controlled by ChemStation A.09.01 (both Agilent Technologies, Waldbronn, Germany). Peptides were eluted with a linear gradient of 5% to 50% acetonitrile (ACN) in 0.1% trifluoroacetic acid with a flow rate of 180 $\mu\text{L}/\text{min}$ for 60 min and lyophilized. Yields were calculated based on peak volumes.

Phosphoramidate-coupled PC-BSA was synthesized by activation of phosphorylcholine (15 μmol ; free acid) with equimolar amounts of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma) in 0.5 mL water and incubation at room temperature (RT) for 30 min. For derivatization 15 nmol of BSA was added to the EDC-activated PC and incubated for 1 h at room temperature [44]. The phosphoramidate-coupled PC-BSA was desalted by NAP5 Columns (GE Healthcare, Freiburg, Germany).

Characterization of PC-modified BSA

For Western-blotting, proteins were separated on a 15% SDS-polyacrylamide gel, transferred to a PVDF membrane by semi-dry blotting, and after blocking with Roti-Block (Roth, Karlsruhe, Germany) the membrane was incubated with the PC-specific antibody TEPC-15 (Sigma, 1:3000 dilution) overnight at 4°C in Roti-Block. Afterwards, the membrane was washed four times for 10 min with PBS-T (PBS containing 0.5% Tween20), incubated with horseradish peroxidase-conjugated anti-mouse Ig (Dako Cytomation, Hamburg, Germany, 1:3000 dilution) for 1 h at RT, and again washed four times for 10 min with Roti-Block. Proteins recognized by the antibody were visualized by enhanced chemiluminescence using the ECL SuperSignal kit (GE Healthcare).

Tryptic Digest

Coomassie stained protein bands from 1D-gels containing up to 30 pmol of protein were excised and transferred into 100 μL

distilled water in LoBind tubes (Eppendorf, Hamburg, Germany). The gel material was washed 15 min under shaking with 100 μ L 50 mM ammonium hydrogen carbonate and destained in the same buffer containing 50% ACN. After removal of solvent, the gel plugs were shrunk twice by addition of 30 μ L ACN with intermediate addition of 50 μ L 50 mM ammonium hydrogen carbonate. Finally, the gel pieces were dried in a vacuum centrifuge (Savant SpeedVac; GMI Jouan, München, Germany). Reduction was performed by incubation with 50 μ L dithioerythritol solution (10 mM in 50 mM ammonium hydrogen carbonate) for 30 min at room temperature. The supernatant was removed and 50 μ L of iodoacetamide (55 mM in 50 mM ammonium hydrogen carbonate) were added. After incubation for 30 min at room temperature, the gel was washed with 100 μ L 50 mM ammonium hydrogen carbonate. Gel plugs were shrunk twice by addition of 30 μ L ACN with intermediate addition of 50 μ L 50 mM ammonium hydrogen carbonate and dried in a vacuum centrifuge. The samples were digested overnight at 37°C in 50 μ L 25 mM ammonium hydrogen carbonate containing 0.5 μ g trypsin (sequencing grade; Promega, Mannheim, Germany). Peptides were recovered by washing the gel material with 10 μ L 0.1% trifluoroacetic acid and stored at -20°C until further use.

MALDI-TOF-MS

MALDI-TOF-MS was performed on an Ultraflex I TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser and a LIFT-MS/MS facility. The instrument was operated in the positive ion reflectron mode. The matrix consisted of 2,5-dihydroxybenzoic acid (DHB, 5 mg/mL; Sigma) supplemented with methylenediphosphonic acid (5 mg/mL; Fluka, Seelze, Germany) in 0.1% TFA. Sum spectra consisting of 300–500 single spectra were acquired. For instrument control, data processing, and data analysis the Compass 1.3 software package consisting of FlexControl 2.4, FlexAnalysis 3.0, and BioTools 3.0 (Bruker Daltonics) was used.

Choline Quantification

PC-residues were removed from PC-BSA by cleavage of the phosphoester bonds with hydrogen fluoride: 10 mg (151 nmol) of lyophilized PC-BSA was dissolved in 500 μ L of hydrofluoric acid (48%, Merck) and incubated on ice overnight. The sample was dried under a stream of nitrogen, dissolved in 500 μ L of water, and lyophilized three times. Choline was measured by HPLC according to published methods [46].

Analysis of a PC-Derivatized BSA Digest

A tryptic in-gel digest of PC-labeled of bovine serum albumin was separated on a Tempo 1D nanoLC system (AB SCIEX, Darmstadt, Germany). A nominal amount of 500 fmol starting material was loaded onto a Reversed Phase-C18 (RP-C18) trap column (Acclaim Pepmap100-C18, 5 μ m particle size, 300 μ m i.d., 5 mm length; Dionex, Thermo Scientific, Dreieich,

Germany) and desalted using 100 μ L of 0.5% (vol/vol) formic acid (FA)/2% (vol/vol) ACN. Separation was achieved by back-flush gradient elution onto a RP-C18 microcolumn (Acclaim Pepmap100 C18, 3 μ m particle size, 75 μ m i.d., 150 mm length) using a 30 min linear gradient of 5%–45% (vol/vol) ACN/0.1% (vol/vol) FA, 250 nL/min. The eluent was analyzed on a 4000 Q TRAP LC/MS/MS system equipped with a Nanospray II source and operated under Analyst 1.4.1 software (all AB SCIEX). Data visualization and annotation were performed using Bioanalyst 1.4.1 and PeakView 2.1 software (AB SCIEX).

Precursor Ion Scanning Experiments

PC-modified peptides were selectively detected using two consecutive precursor ion scanning experiments per cycle for m/z 104.1 (choline C₅H₁₄NO) and m/z 184.1 (phosphorylcholine C₅H₁₅NO₄P) in positive ion mode, respectively. For each precursor ion scan, m/z range 400–1200 was scanned using a scan time of 2.5 s and fixed collision energies of 55 and 50 eV, respectively. Up to the five most abundant signals detected in either precursor ion scan were then submitted to a high-resolution linear ion-trap MS scan to determine the corresponding monoisotopic mass, charge state, and molecular weight, resulting in a maximum cycle time of 6 s.

MRM Optimization of Collision Energy

MRM transitions were set up in positive ion mode for nine putative PC-modified precursors showing a response in both the m/z 104 and 184 precursor ion scans. For each precursor/fragment pair, six transitions were used at collision energies stepped from 30 to 80 eV in steps of 10 eV, resulting in a total of 108 transitions, which were monitored in two separate runs with 54 transitions each using a dwell time of 40 ms and unit resolution (0.7 FWHM) in both quadrupoles. In both runs, MRM transitions showing threshold intensity were confirmed by data-dependent high resolution linear ion trap MS scans, and Enhanced Product Ion (EPI) MS/MS scans employing Q2 collision-cell activation using rolling collision energy, nitrogen as collision gas, and recording of the fragments at a scan speed of 4000 Da s⁻¹ in the Q3 linear ion trap. For each of the nine putative precursors, the best collision energy was selected; averaging across precursors provided best values of +55 eV for transitions to m/z 104.1 and +50 eV for transitions to m/z 184.1, respectively.

MRM-Triggered MS/MS Experiments

For identification and site localization, MRM transitions for 17 putative PC-modified peptide precursors identified in the precursor ion scanning experiment to m/z 104.1 and 184.1 were monitored, resulting in a total of 34 transitions (Table 1). Q1 masses were in the range of m/z 440–690 for doubly charged precursors, and in the range of m/z 410–660 for triply charged precursors. MRM transitions were designed using the optimized collision energies of 55 and 50 eV, respectively, unit

Table 1. 34 MRM transitions for 17 candidate PC-peptides derived from precursor ion scan experiments of a tryptic digest of PC-modified BSA

Q1	Q3	CE [eV]	Dwell time [ms]	Identified by MS/MS
684.3	184.1	50	50	No
684.3	104.1	55	50	No
606.8	184.1	50	50	No
606.8	104.1	55	50	No
652.2	184.1	50	50	CCTESLVNR
652.2	104.1	55	50	CCTESLVNR
654.0	184.1	50	50	RKVPQVSTPTLVEVSR
654.0	104.1	55	50	RKVPQVSTPTLVEVSR
477.8	184.1	50	50	No
477.8	104.1	55	50	No
546.8	184.1	50	50	YLYEIAR
546.8	104.1	55	50	YLYEIAR
590.3	184.1	50	50	QTALVELLK
590.3	104.1	55	50	QTALVELLK
548.8	184.1	50	50	No
548.8	104.1	55	50	No
603.3	184.1	50	50	No
603.3	104.1	55	50	No
487.3	184.1	50	50	No
487.3	104.1	55	50	No
449.8	184.1	50	50	No
449.8	104.1	55	50	No
416.6	184.1	50	50	No
416.6	104.1	55	50	No
602.3	184.1	50	50	KVPQVSTPTLVEVSR
602.3	104.1	55	50	KVPQVSTPTLVEVSR
535.7	184.1	50	50	No
535.7	104.1	55	50	No
445.6	184.1	50	50	No
445.6	104.1	55	50	No
667.9	184.1	50	50	No
667.9	104.1	55	50	No
584.3	184.1	50	50	LVVSTQTALA
584.3	104.1	55	50	LVVSTQTALA

resolution in both quadrupoles and dwell times of 50 ms. On exceeding a threshold intensity of 2500 cps, up to two precursors were confirmed using high resolution linear ion trap MS and enhanced product ion MS/MS scans.

Identification of PC-Modified Proteins by Sequence Database Searching

Product ion spectra from the MRM-triggered MS/MS experiments were searched against the complete *Bos taurus* proteome UniProt sequence database (revision 08/14, 24139 entries) supplemented by 50 common lab contaminants using ProteinPilot 4.5.0.0 software (Build 1656; AB SCIEX). N-/O-/S-modification of phosphorylcholine was introduced to the software's data dictionary and added to the "Special Factors: Gel-based ID" modification set at a feature probability of 49%, reflecting the specificity of the MRM detection.

Detection of PC-Modified Peptides in a Full Proteome Digest

PC-labeled peptides (PC coupled as amidate and azophenyl to CS-01) were dissolved in 10 mM ammonium formate to a final concentration of 2 pmol/ μ L and analyzed either alone or spiked

into a total digest of *Arabidopsis thaliana* (500 pmol of each synthetic peptide and 1 μ g *A. thaliana* digest, respectively). Peptides were separated by an HPLC system (Eksigent ekspert nanoLC 400 system, AB SCIEX, Darmstadt, Germany) connected to a QTRAP 4500 LC/MS/MS system equipped with a NanoSpray III source and operated under Analyst 1.6 software. HPLC was performed on a C18 column (Acclaim Pepmap100 C18, 2 μ m particle size, 75 μ m i.d., 250 mm length; Dionex, Thermo Scientific). A linear gradient of 10% to 30% ACN in 0.1% formic acid was applied to elute the peptides at a flow rate of 300 nL/min within 60 min. All mass spectrometry measurements were done in nano positive ion spray mode (ion spray voltage 2800 V). The modified peptides could be first detected by precursor ion scanning for the typical fragments choline and phosphorylcholine (m/z 104.1 and 184.1, respectively), which triggers an enhanced product ion scan (MS/MS) in the linear ion trap for peptide identification (scan speed 10000 Da s^{-1}). The precursor ion scan was done with a Q1 scan speed of 1000 Da s^{-1} , the collision energy was ramped between 70–90 eV for fragment m/z 104.1 and between 50 and 70 eV for fragment m/z 184.1. Additional data were acquired by MRM-triggered enhanced product ion (EPI) scans to further verify the results of the first experiment. The MRM was built by the detected or calculated phosphorylcholine modified peptide precursors mass (Q1 mass) and the specific fragments m/z 104.1 and 184.1 Da (Q3 mass). A positive MRM signal triggered an Enhanced Product Ion MS/MS scan with fragment detection in the linear ion trap. Data were analyzed with PeakView 2.1 (both from AB SCIEX).

Results and Discussion

Synthesis and Purification of PC-substituted Peptides and Proteins, and Characterization of the Products by MALDI-TOF-MS

Essentially, two one-step methods have been described for the synthesis of PC-modified peptides and proteins: first, PC becomes activated by a carbodiimide-derivative and is subsequently coupled to the side chains of polar amino acids, resulting in phosphoamidate- (lysine) and phosphoester- (serine, threonine, and tyrosine) linkages (Figure 1a). A second method is the reaction of *p*-aminophenyl-PC via activation as diazonium-derivative to the side chains of tyrosine and histidine (Figure 1b). Furthermore, this diazonium-derivative can also react with lysine. However, in case of the ϵ -aminogroup of lysine as an acceptor, the resulting linkage is not a diazo-group but a secondary amine-bond and referred to as PC* in the following (Figure 1b). For PC-modification we used peptides that were amidated at their C-terminus, to avoid carboxyl groups for the phosphoamidate method, which would otherwise result in carbodiimide-catalyzed polymerization of the peptide.

For PC-derivatization, we used a synthetic peptide (CS-01; PVQNKFFGLA-amide) lacking a free primary amino-group at the N-terminus, a free C-terminus, and other functional groups

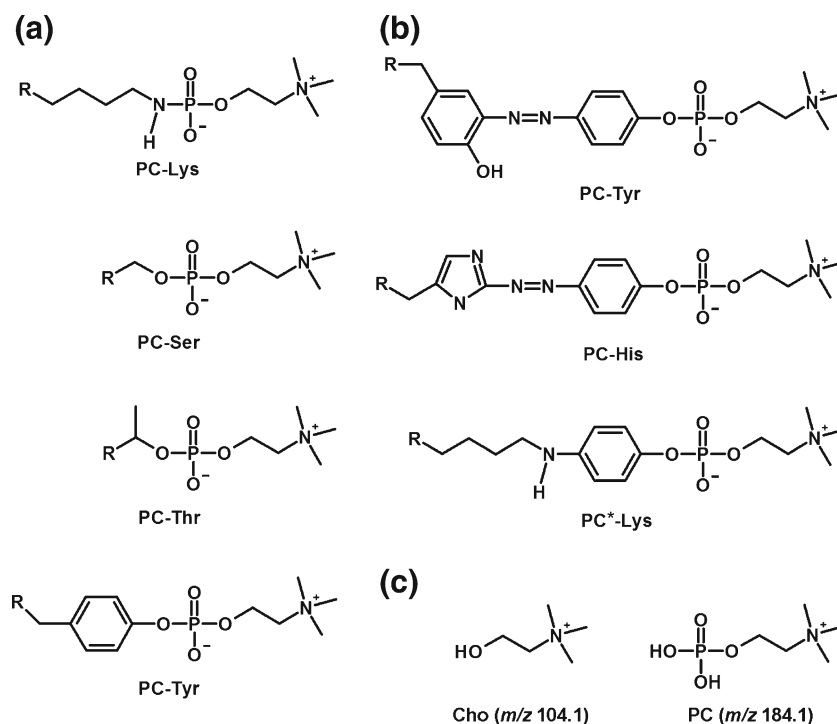


Figure 1. Synthetic PC-epitope structures generated by phosphoamidate- (a) or diazo coupling (b). Structures from carbodiimide-chemistry resulted in phosphoamidate- (Lys) or phosphoester- (Ser, Thr, Tyr) linkages to the peptide backbone (a), whereas diazo-coupling resulted in modifications of Tyr, His and Lys (b). The side-chain of the modified amino acid is shown and R represents the rest of the peptide or protein. PC*: in case of diazo-coupling to Lys, the resulting molecule contains a secondary amine instead of a diazo-group. (c) Structures of the marker ions choline (m/z 104.1) and phosphorylcholine (m/z 184.1) characteristic for the fragmentation of PC-substituted peptides

at the side chains to prevent extensive side reactions. Derivatization of the peptide CS-01 ($[M + H]^+$ observed at m/z 1119.5) with both methods resulted in products named as CS-01-PC (phosphoamidate-coupling; $[M + H]^+$ at m/z 1284.6 Da, Figure 2a) and CS-01-PC* (diazo-coupling; $[M + H]^+$ at m/z 1376.7, Figure 2c) with yields of 62% and 10%, respectively, as determined by the HPLC-purification.

The two different PC-modified peptides were analyzed by MALDI-MS/MS. The spectra (Figure 2b and d) showed strong signals for the fragments of choline (m/z 104.1) and phosphorylcholine (m/z 184.1; Figure 1c) as well as fragment ions resulting from the neutral loss of 59 Da, attributable to a Hoffmann elimination of trimethylamine from the choline moiety and formation of a cyclic phosphodiester [43]. Especially in the case of the phosphoamidate, the preferential fragmentation of the PC moiety lead to poor fragmentation of the peptide backbone, resulting in weak signals of the a-, b-, and y-ion series in the MS/MS spectra (Figure 2b). This situation parallels the observation of the fragment ion series containing dehydroalanine when CID-induced phosphoric acid loss from phosphoserine has taken place [47]. The diazo-coupled peptide exhibited less Hoffman elimination; here the fragments cover the whole sequence (Figure 2d). Nevertheless, for both peptides the PC-substituent could be unambiguously localized to the lysine residue (assigned as K* in Figure 2b and d), by b4 and b5 (m/z 439.3 and 732.8, respectively) ions in the case of

CS-01-PC (Figure 2b) and a4, a5, b4, and b5 (m/z 411.3, 795.6, 438.6, and 823.8, respectively) in the case of the CS-01-PC* (Figure 2d).

These investigations clearly demonstrated the dominant influence of PC-substituents on the mass spectrometric fragmentation. The neutral loss of the trimethylamine group (M-59) resulted in cyclic phosphate derivatives. This Hoffmann elimination was also found for trimethylated lysine residues of histones and acylcarnitines [25, 26]. The cyclic phosphate derivative formed in the case of PC-substituents seems to trap the positive charge very strongly, thus suppressing the formation of peptide specific fragment ions. In contrast, fragmentation of the peptide backbone was not affected in the case of the tri-*N*-methylated lysines in histones since respective cyclic phosphate derivatives were not formed in that case. When ion trap instruments are used, ion traces of m/z 104.1 and m/z 184.1 can hardly be used because low mass ions are often absent or of low intensity. Here, a neutral loss scan (M-59) can be used to screen complex peptide mixtures for the presence of PC-substitutions for a selection of these peptides for MS/MS analyses [43].

As a third and more complex derivative, we modified BSA with PC using the phosphoamidate chemistry. The coupling was verified by a Western blot experiment, detecting the PC-modification with the PC-specific antibody TEPC-15 (Figure 2e). After cleavage of the phosphodiester bonds with hydrogen

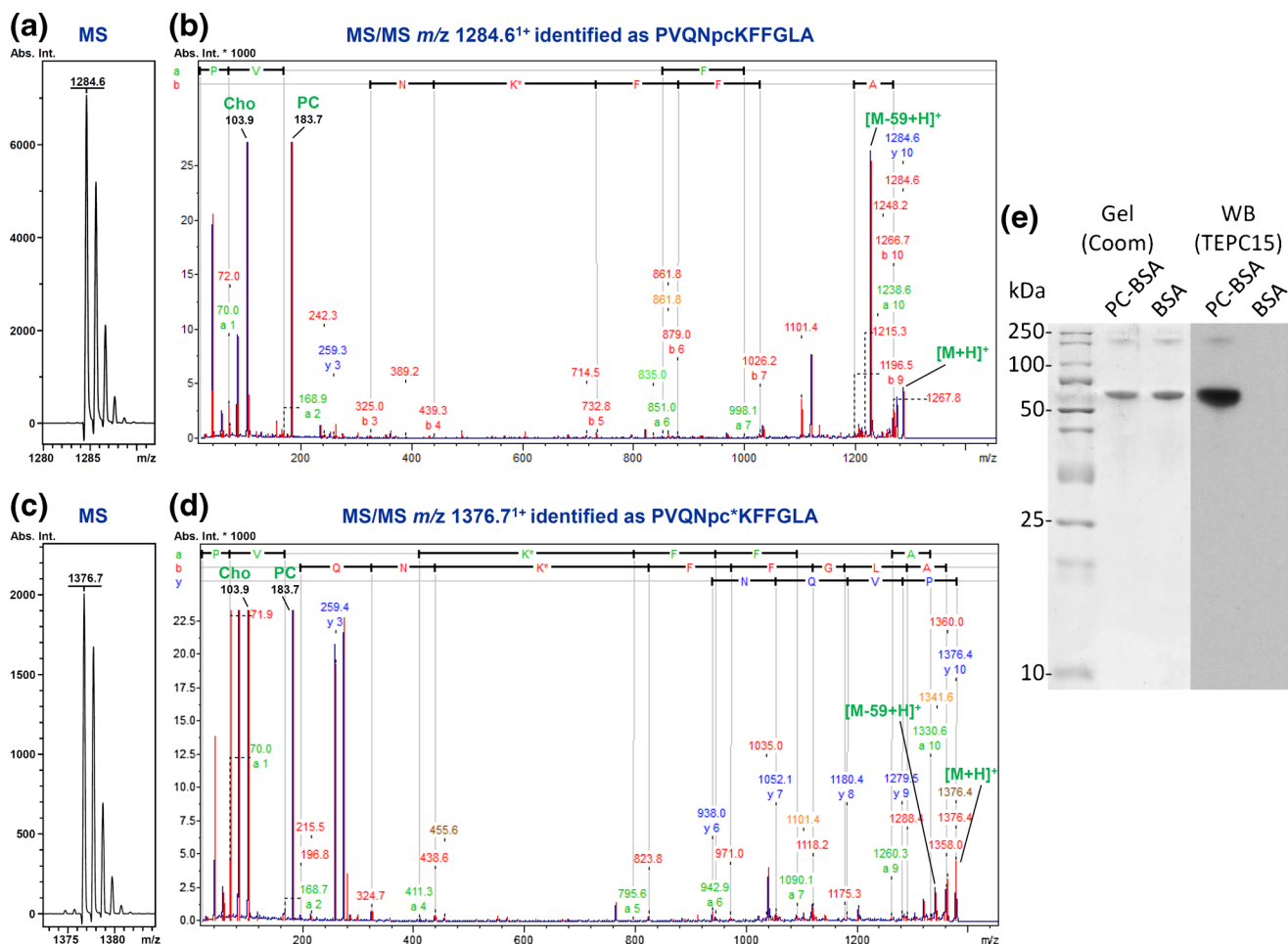


Figure 2. Characterization of synthetic PC-peptides and proteins by MALDI-TOF-MS and Western blot. MALDI-TOF-MS and MS/MS spectra of the PC-modified peptide CS-01 (a)–(d). The MS/MS spectra of the phosphoamidate- (b) and the diazo-derivative (d) show strong signals for choline (m/z 104.1) and PC (m/z 184.1). In addition to the $[M + H]^+$ ions (m/z 1284.6 and 1376.7), fragment ions resulting from the neutral loss of 59 Da (Hoffman elimination of trimethylamine) were observed. (e) The Coomassie-stained gel with PC-BSA and unmodified BSA and the respective Western blot with the PC-specific antibody TEPC-15 showing a strong positive signal in case of PC-BSA

fluoride, a substitution of 591 pmol PC per 151 pmol BSA—giving a molar ratio of 3.9—could be determined by quantifying the amount of choline released as described in [46]. For analysis by MRM-triggered MS, we digested the PC-BSA with trypsin, yielding a complex but defined mixture of PC-modified and unmodified peptides.

Development of a LC/MS/MS Workflow for the Detection and Localization of PC-Substituents

Although the detection and localization of PC with MALDI-MS/MS works well for single peptides, this method was found to be not suitable for the analysis of complex peptide mixtures from proteolytic digests of PC-modified proteins where usually only a small number of peptides carries the modification in sub stoichiometric amounts.

Based on the observation that MS/MS-fragmentation of PC-modified peptides usually generates strong signals for choline (m/z 104.1) and phosphorylcholine (m/z 184.1), we developed

a two-stage workflow for the detection and localization of PC-modifications in peptides by nanoLC/MS/MS on hybrid triple quadrupole/linear ion trap (QTRAP) mass spectrometers.

MRM-triggered MS/MS acquisition has been previously described as a powerful tool for the detection of posttranslational protein modifications such as phosphorylation [48, 49], acetylation [50], and ubiquitination [51]. In the so-called MIDAS approach (MRM-initiated detection and sequencing), the MRM Q1 masses corresponding to the modified peptide are usually predicted theoretically if both an exact protein sequence and the specificity of the modification are known. Especially for less site-specific or heterogeneous modifications, a modification-specific precursor ion scan can be used to confine the otherwise rapidly expanding list of potential precursor masses in a scouting experiment. This two-stage workflow has been described for the successful analysis of covalent peptide-RNA crosslinks [52]. Applying this approach to the detection of PC-modified peptides, we here used two consecutive precursor ion scans for the m/z 104.1/184.1 marker ions to

detect potential PC-peptide candidates. Data-dependent high resolution linear ion trap scans were used to determine the charge states and molecular masses of these candidates (Figure 3, top). In a second experiment, MRM transitions from the detected candidates' m/z to the m/z 104.1/184.1 marker ions were used to trigger MS/MS experiments under optimal conditions (i.e., close to the LC peak apex) (Figure 3, bottom). In addition, an overlay of the candidates' m/z 104.1 and m/z 184.1 traces should be used to confirm the presence of the PC modification on the peptide.

Optimization of CID-Fragmentation Conditions for the Detection of PC-Substituted Peptides

The CID collision energy used for precursor ion scanning and MRM were optimized using candidate peptides from the PC-BSA digest. Production of the marker ions as a function of collision energy was monitored using six MRM transitions per precursor and fragment with collision energies ranging from 30 to 80 eV in steps of 10 eV. By observing the best values and averaging across nine precursors, best values of 55 and 50 eV were found as the optimum collision energy values for production of m/z 104.1 and 184.1, respectively (Figure 4).

Application of the LC/MS/MS Workflow for the Detection and Localization of PC-Substituted Peptides within a Model Tryptic Digest of PC-BSA

In the next experiment, we applied the optimized settings to identify PC-substituted peptides within a complex peptide mixture and to localize the PC-modification within the peptide sequence using MRM-triggered collision cell CID MS/MS spectra. From the previous precursor ion scan experiments, 17 candidate precursor ions showing response both in the m/z 104.1 and 184.1 precursor ion scans were selected and targeted by MRM transitions using the optimized collision energy regime (Table 1, Figure 5a). High resolution linear ion trap MS spectra as well as collision cell CID-MS/MS spectra that were triggered from MRM channels exceeding threshold intensity were acquired to achieve peptide identification and site localization.

A sequence database search including phosphorylcholine as a variable N-/O-/S- modification against the complete *Bos taurus* proteome returned only the target protein BSA at >95% confidence, reflecting the specificity of the targeted MRM detection and the quality of the product ion spectra. From the MRM-triggered MS/MS spectra, various PC-modified BSA peptides could be confidently assigned by sequence database searching and manual review (Table 2).

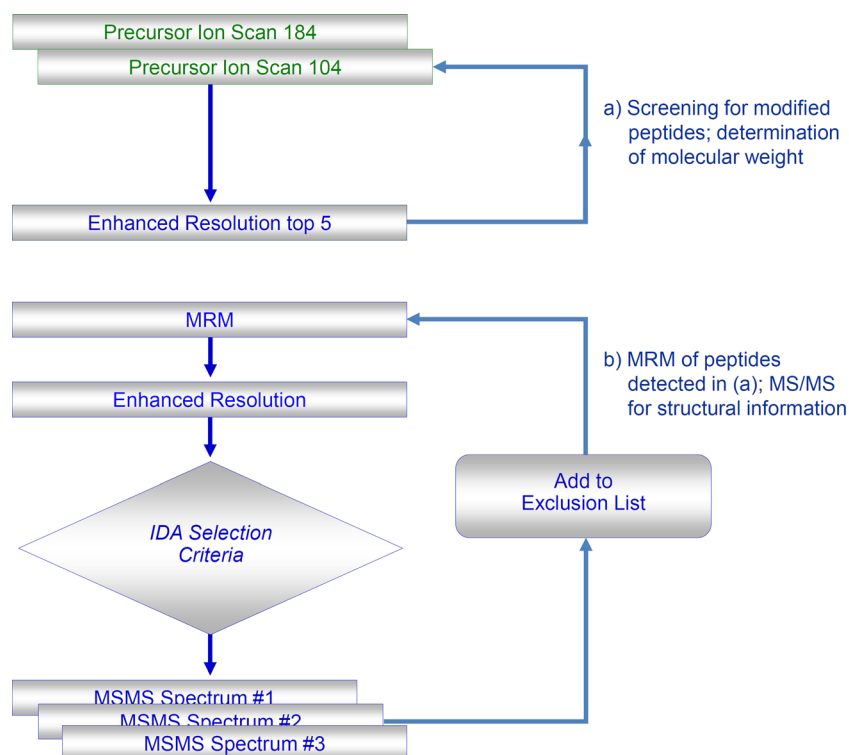


Figure 3. LC/MS/MS workflow for the detection and localization of PC-modifications. In a first HPLC run precursor ion scans for choline (m/z 104 Da) and phosphorylcholine (m/z 184 Da) generated under optimized CID-conditions were used to detect PC-modified peptides, followed by a high resolution linear ion trap MS-scan to determine the charge state and the molecular weight of the PC-modified precursor. In a second experiment, MRM-triggered MS/MS spectra of these precursors were recorded under optimal conditions. IDA = information-dependent acquisition

Ramping of collision energy for marker ion production (m/z 184.1) from 30 to 80 eV

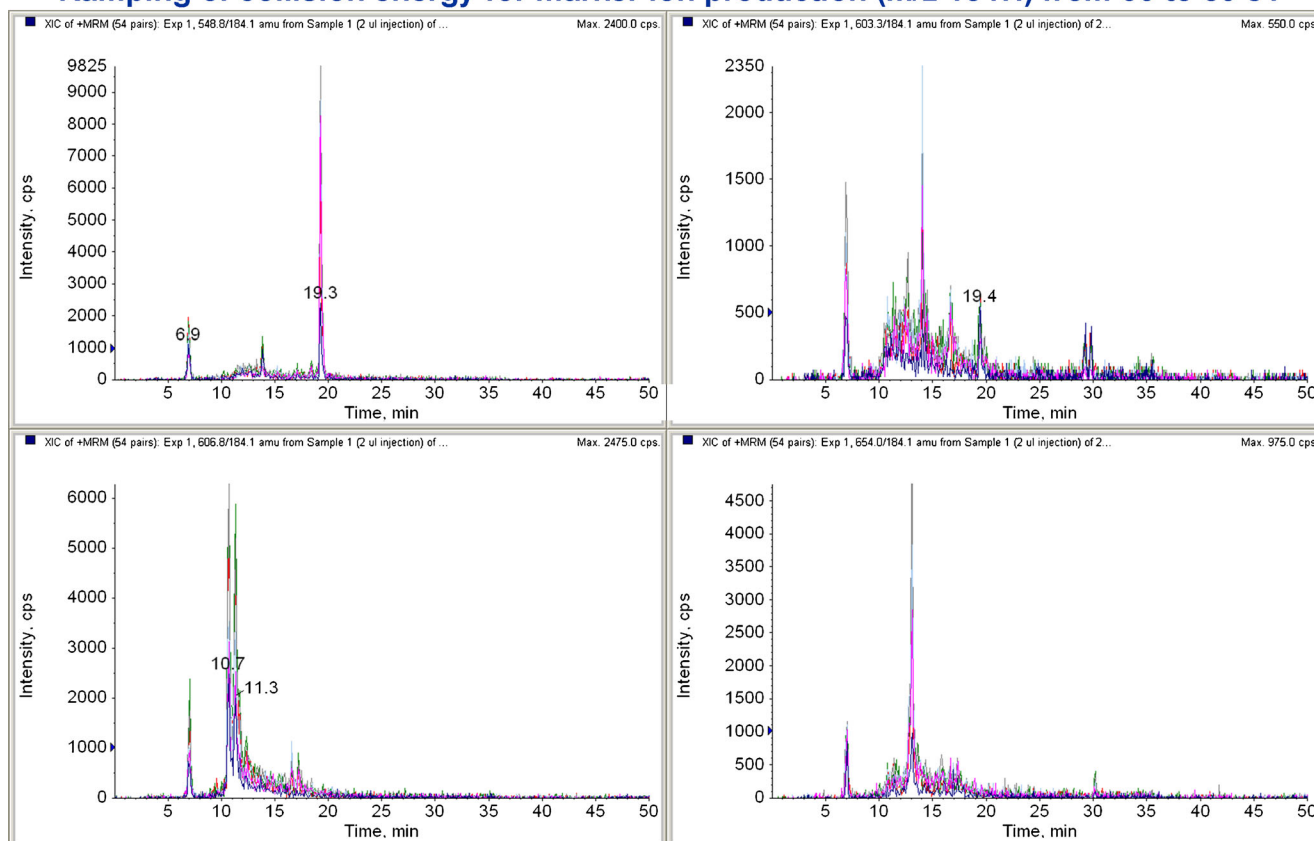


Figure 4. Parameter optimization for the detection and fragmentation of PC-peptides. The CID collision energy used for precursor ion scanning and MRM-triggered fragmentation was optimized using four candidate peptides from a tryptic digestion of PC-BSA. Collision energies of 55 and 50 eV, respectively, were found as optimal for the production of m/z 104 and 184 fragments and fragmentation

Markedly, only one non-PC-modified peptide was identified. Figure 5b–e show four examples for peptides carrying PC epitopes on Lys, Tyr, and Thr, respectively. Most of the CID spectra obtained showed strong fragments that could be attributed to the neutral loss of choline or the complete phosphorylcholine moiety, i.e., $[M + H - 103]^+$ (loss of choline) and $[M + H - 183]^+$ (loss of phosphorylcholine), plus abundant signals for the marker ions for choline (m/z 104.1) and phosphorylcholine (m/z 184.1). This fragmentation pattern confirmed the PC-substitution. Lys-derivatized peptides show a higher relative intensity of sequence-specific b- and y-type fragment ions versus the marker ions, indicating a higher stability of the N-derivatization under CID conditions compared to the O-derivatization in PC-Tyr/PC-Thr-epitopes. In comparison to the MALDI-MS/MS analyses shown above, it seemed that the Hoffmann elimination was reduced under these experimental conditions; therefore, more sequence-specific fragment ions were generated, which facilitates sequence identification and localization of the PC modification site. Additional confirmation for the PC-substitution can be drawn from the MRM intensity ratios for the m/z 104 and 184 transitions of each precursor, respectively. For the successfully identified peptides, we consistently observed 104/184 intensity ratios

between 3/1 and 10/1, irrespective of the modified residue, precursor m/z , or position of the modified residue.

Besides the seven PC-modified peptides identified in the experiment, more high quality MS/MS data were acquired for the other targeted precursors; however, no further sequences could be assigned. We believe that this is a consequence of the presence of abundant fragment ions in the MS/MS spectra that are specific for loss of the PC modification rather the peptide sequence, especially for O-modified peptides. As non-sequence-specific fragments do not contribute to the scoring, this would also explain the partially low confidence scores assigned by the Paragon algorithm to the identified peptides (Table 2). Improvements on the scoring function or the use of alternative fragmentation techniques might be used to improve this in future experiments.

Detection and Localization of PC-Substituted Peptides within a Complex Tryptic Digest of Arabidopsis thaliana

In this experiment, we tested the detection of PC-modified peptides in a more realistic proteomic situation: The two PC-peptides CS-01-PC and CS-01-PC* where spiked into the total

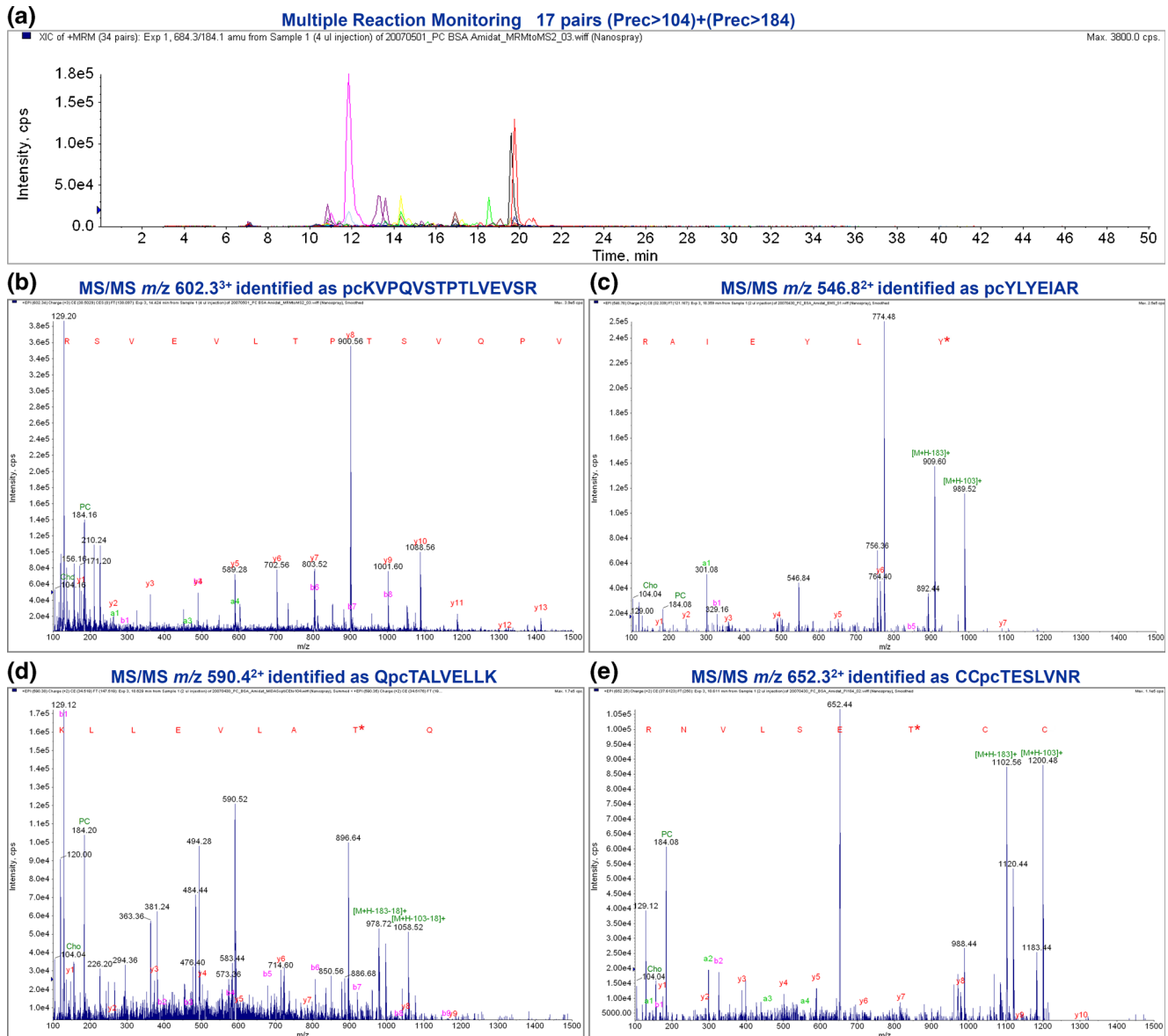


Figure 5. MIDAS-LC/MS/MS MRM profile of the tryptic digest of PC-BSA and selected MS/MS spectra from PC-substituted peptides. (a) Precursor ion scan profiles of the tryptic digest of PC-BSA for precursor ions giving fragments for choline (m/z 104.1) and phosphorylcholine (m/z 184.1). (b)–(e) MS/MS-spectra of selected PC-modified peptides from PC-BSA found in the precursor ions scans. Spectra are characterized by strong fragment ion signals resulting from the loss of the choline or phosphorylcholine moieties ($[M + H - 103]^+$ or $[M + H - 183]^+$) and the marker ions m/z 104.1 and 184.1 Da. The fragmentation patterns allowed an unambiguous localization of the PC-modification within the sequences

tryptic digest of *Arabidopsis thaliana* (500 pmol of each synthetic peptide and 1 μ g *A. thaliana* digest, respectively) and analyzed with the AB SCIEX QTRAP 4500 LC/MS/MS system, the successor of the QTRAP4000. The QTRAP 4500 is characterized by an at least 3-fold increased sensitivity and faster scanning capabilities.

By adjusting the settings from the QTRAP 4000 to the QTRAP 4500, we obtained CID spectra triggered by the MRM traces, which we could unambiguously assign to the two PC-substituted peptides. Figure 6a shows the total ion chromatogram with the CS-01-PC* (azophenyl-PC derivative) and the CS-01-PC (PC-amidate derivative) eluting

at 34.46 min and 45.04 min, respectively. Figure 6b and c display the MIDAS-profiles with the simultaneous occurrence of the marker fragments for choline and phosphorylcholine (m/z 104.1 and 184.1 Da) at the time points indicated above. The corresponding CID spectra showed strong fragments that could be assigned to single- and double-charged b- and y-fragment ions, with the double-charged fragment ions displaying higher intensities (Figure 6d, E; b²⁺-ions shown in cyan, y²⁺-ions shown in purple). In both peptides, the PC-substituent could be unambiguously allocated to the lysine residue (assigned as K* in Figure 6d and e). Indicative ions were the b₄, b₅, y₅, and y₆ fragments (m/z 439.23, 824.49,

Table 2. Peptides Identified by Sequence Database Searching from an MRM-Triggered MS/MS Analysis of a Tryptic Digest of PC-Modified BSA

N	Conf. ¹ [%]	Sequence	Modifications ²	Non-tryptic cleavages	RT [min]	z	<i>m/z</i> (exp.)	<i>m/z</i> (theor.)	Δ <i>m</i> [Da]	MW (theor.) [Da]
1	96.5	KVPQVSTPTLVEVSR	PC(K)@1		14.4	3	602.32	602.34	0.02	1803.99
2	31.9	QTALVELLK	PC(T)@2		19.0	2	590.34	590.34	0.00	1178.67
3	1.1	RKVQVSTPTLVEVSR		cleaved T-R@N-term	14.3	3	599.95	599.35	0.60	1795.03
4	0.6	LVVSTQTALA	PC(S)@4		20.5	2	583.82	584.32	0.50	1166.63
5	0.5	YLYEIAR	PC(Y)@1		18.5	2	546.80	546.78	0.02	1091.54
7	0.2	CTTESLVNR	Cam(C)@1 Cam(C)@2 PC(T)@3		11.1	2	652.26	652.28	0.02	1302.55
8	0.2	RKVQVSTPTLVEVSR	PC(K)@2	cleaved T-R@N-term	13.3	3	654.02	654.37	0.35	1960.09

¹Confidence score allocated by the Paragon algorithm in the ProteinPilot software.

²Variable modifications abbreviated as PC (phosphorylcholine), Cam (carbamidomethyl).

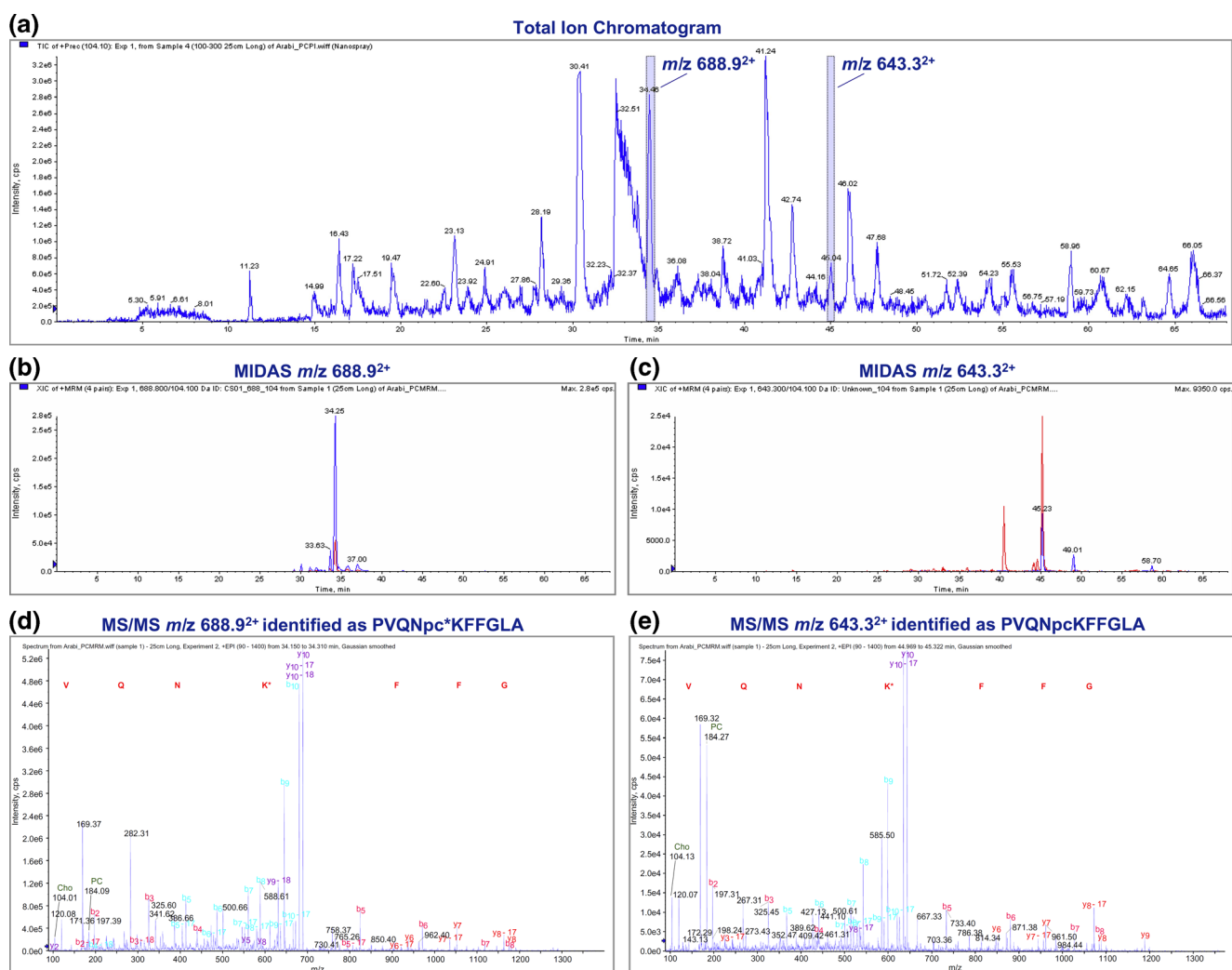


Figure 6. Detection of PC-substituted peptides in a complete tryptic digest of *Arabidopsis thaliana* and localization of the PC-modification within the peptide sequence by MS/MS. (a) Total ion chromatogram of a tryptic digest of *A. thaliana* with the two PC-peptides spiked in. By MRM of the two indicative ions for choline (*m/z* 104.1) and phosphorylcholine (*m/z* 184.1) both peptides could be detected (shaded areas at 34.46 and 45.04 min); (b) and (c) MIDAS-profiles for the precursor fragment ions of 104.1 (blue) and 184.1 (red); (d) and (e) MS/MS-spectra of the PC-modified peptides. The spectra are characterized by strong fragment ion signals of the b- and y-series, both found as single and double charged ions and the marker ions *m/z* 104.1 and 184.1. Assigned fragment ions are shown in the following colors: b⁺: magenta, y⁺: red, b²⁺: cyan, and y²⁺: purple. The fragmentation patterns allowed an unambiguous localization of the PC-modification within the amino acid sequences

553.31, and 938.57 Da, respectively) in case of CS-01-PC* (Figure 6d), and the b4 and y5 ions (m/z 439.23, and 733.47 Da, respectively) in case of CS-01-PC (Figure 6e). For the double-charged fragment-ions, numerous additional signals resulting from the loss of ammonia (-17 Da) could be observed (for clearer arrangement not annotated in Figure 6). Interestingly, no signals originating from the neutral loss of choline or the complete phosphorylcholine moiety, i.e., $[M + H - 103]^+$ (loss of choline) and $[M + H - 183]^+$ (loss of phosphorylcholine), or $[M+H - 59]^+$ (Hoffmann elimination of trimethylamine) could be observed. This is consistent with the fragmentation behavior observed for the PC-BSA peptides, where N-derivatized peptides also produced predominantly sequence-specific fragments, whereas the spectra of O-derivatized peptides exhibited significant production of the $[M + H - 103]^+$ and $[M + H - 183]^+$ fragments, indicating a higher stability of the phosphoamidate bond under these conditions (compare Figure 5b–d).

Conclusion

Using a hybrid triple quadrupole/linear ion trap mass spectrometer, we have developed a LC/MS/MS workflow that allowed for the first time a sensitive and selective detection of PC-modified peptides within complex proteolytic digests. Precursor ion scanning and MRM of marker fragments m/z 104.1 and 184.1 were used to trigger collision cell CID fragmentations of modified peptides, allowing the identification of the peptide backbone and the localization of the PC-substituent. The transfer of the workflow from the QTRAP 4000 to the more sensitive QTRAP 4500 allowed us to detect and confirm PC-modified peptides even within a highly complex background of a tryptic digest from a complete organism. This methodological development is the prerequisite to head for the identification and structural determination of naturally occurring PC-epitopes from parasites.

Acknowledgments

The authors thank Michael Dreisbach, Christina Schmidt, and Hans-Günter Welker for excellent technical assistance. This work was supported by the German Research Council (SFB 535, TP Z1), the Rhön-Klinikums AG, and the LOEWE Research Focus “Non-neuronal cholinergic systems,” an interdisciplinary collaborative research effort funded by the State of Hessen.

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