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Identification of Phosphoserine and Phosphothreonine as Cysteic Acid and β-Methylcysteic Acid Residues in Peptides by Tandem Mass Spectrometric Sequencing

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Tandem mass spectrometry has long been an intrinsic tool to determine phosphorylation sites in proteins. However, loss of the phosphate moiety from both phosphoserine and phosphothreonine residues in low-energy collision-induced dissociation is a common phenomenon, which makes identification of P-Ser and P-Thr residues complicated. A method for direct sequencing of the Ser and Thr phosphorylation sites by ESI tandem mass spectrometry following β-elimination/sulfite addition to convert $-HPO_4$ to $-SO_3$ has been studied. Five model phosphopeptides, including three synthetic P-Ser-, P-Thr-, or P-Ser- and P-Thr-containing peptides; a protein kinases C-phosphorylated peptide; and a phosphopeptide derived from β-casein trypsin digests were modified and then sequenced using an ESI-quadrupole ion trap mass spectrometer. Following incubation of P-Ser- or P-Thr-containing peptides with Na_2SO_3/NaOH, 90% P-Ser and 80% P-Thr was converted to cysteic acid and β-methylcysteic acid, respectively, as revealed by amino acid analysis. The conversion can be carried out at 1 μM concentration of the peptide. Both cysteic acid and β-methylcysteic acid residues in the sequence were shown to be stable and easily identifiable under general conditions for tandem mass spectrometric sequencing applicable to common peptides.

Protein phosphorylation on Ser, Thr, and Tyr residues is recognized as the most important reaction in the regulation of cell functions. Serine and threonine phosphorylation of proteins comprise ∼99% of the proteins phosphorylated.[1] Because of the inherent sensitivity and speed of tandem mass spectrometry, this technique has become a preferred choice for identifying phosphorylation sites on phosphopeptides derived from the proteolytic digestion of phosphorylated proteins.[2] The quadrupole ion trap mass spectrometer is widely employed for routine peptide analysis and sequencing.[3,4]Until recently, the most frequently used methodology for identification of phosphorylated amino acid residues has been sequencing the selected phosphopeptide using tandem mass spectrometry with a variety of platforms.[4−6] However, the loss of the phosphate moiety from P-Ser or P-Thr during CID makes identification of the phosphorylation sites difficult, if not impossible.[6] Alternatively, scanning for the neutral loss of phosphoric acid can be utilized for the identification of phosphopeptides.[7−10] However, intrinsic instability of P-Ser and P-Thr even during low-energy collision-induced dissociation results in complicated mass spectra that require special platforms for analysis but still do not allow direct identification of multiple phosphorylated residues in a peptide.

Previously, methods have been reported based on Edman degradation or N-terminal sequencing of phosphopeptides in which the phosphoserine is modified by β-elimination/nucleophile addition.[11−13] Because of less reactivity of P-Thr in the β-elimination step, this methodology does not apply to the identification of phosphothreonine. Furthermore, racemization occurs during the β-elimination/nucleophile addition, and the modified products may not all be identifiable in routine PTH-amino acid analysis

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Table 1. Percent Yields of Cysteic Acid and β-Methylcysteic Acid and Percent Reduction of Ser and Thr after β-Elimination/Sulfite Addition from KMpS and KMSpT Phosphopeptides

	25 °C			37 °C			25 °C			37 °C		
Na_2SO_3 (M)[a]	CA[b]	Ser[b]	*c*	CA[b]	Ser[b]	*c*	mCA[b]	Thr[b]	*c*	mCA[b]	Thr[b]	*c*
0.1	65.3	77.2	(85)	72.2	84.7	(85)	14.2	46.1	(31)	73.6	86.5	(85)
0.3	76.7	83.3	(92)	83.9	85.4	(98)	25.2	51.2	(49)	80.1	85.4	(94)
0.6	82.2	91.6	(90)	89.6	94.0	(95)	33.3	57.4	(59)	82.5	85.3	(97)
1.0	84.4	92.3	(91)	90.5	95.4	(95)	40.1	63.4	(63)	84.5	85.3	(99)

[a] Reactions were carried out in 0.1 N NaOH for 24 h at varied concentrations of Na_2SO_3 as indicated. [b] Amino acid analysis values of cysteic acid (CA), Ser, β-methylcysteic acid (mCA), and Thr, are expressed in molar percent relative to leucine. [c] Numbers in parentheses are values of the CA divided by the reduced Ser or the mCA divided by the reduced Thr of the same reaction condition in percent.

methodology. To facilitate stability upon CID that permits identification of the phosphorylated sites by electrospray tandem mass spectrometry, a method of converting P-Ser and P-Thr into respective (*S*)-ethylcysteine and β-methyl-(*S*)-ethylcysteine by the β-elimination/ethanethiol addition was reported.[14] Suitable data is obtained in the case of phosphoserine-containing peptides; however, the difficulty of converting P-Thr to β-methyl-(*S*)-ethylcysteine has not been overcome in these procedures. In addition, the ethanethiol used in such procedures is extremely foul-smelling and has limited solubility in H_2O, thereby requiring sample cleanup prior to LCMS analysis, since the conversion is carried out in the presence of 37% organic solvent. For the identification of *O*-glycosyl and *O*-phosphoryl sites in proteins, β-elimination and sulfite addition has been utilized, followed by the analysis of cysteic acid, Edman degradation, or ^{35}S-labeling.[15−17] It also has been recognized that Na_2SO_3 addition is more efficient than mercaptoalkane addition in the case of the P-Thr-containing peptides.[18]

Here, we describe an approach for the identification of the serine and threonine phosphorylation sites in peptides by MS/MS fragmentation in an LC-coupled electrospray ion trap mass spectrometer following conversion of the peptide phosphate moiety into cysteic acid and β-methylcysteic acid. We have also devised conditions and demonstrated that both cysteic acid and β-methylcysteic acid residues are readily obtainable from l μM phosphopeptide and identifiable in peptide sequences derived from tandem MS analysis.

EXPERIMENTAL PROCEDURES

Materials. Serine phosphopeptide (KMpSTLSYR), designated KMpS; threonine phosphopeptide (KMSpTLSYR), KMSpT; and serine/threonine diphosphopeptide (KMpSpTLSYR), KMpSpT, were synthesized by Princeton Biomolecules (Langhorne, PA). Purified protein kinases C from rat brain, and PKC substrate peptide, bovine myelin basic protein residues 4−14 (MBP_{4-14}, QKRPSQRSKYL) was purchased from Upstate Biotechnology (Lake Placid, NY). Modified sequencing grade trypsin was from Promega (Madison, WI). $ZipTip_{u-C18}$ was a product of Millipore Corp. (Bedford, MA). Reversed-phase columns, C_{18} 300 Å, 4.6 mm × 250 mm, and 0.3 mm × 50 mm for LC/MS analysis were purchased from Vydac (Hesperia, CA) and Phenomenex (Torrance, CA), respectively. The fused-silica column (Aquasil C_{18} 100 Å) for nanospray injections was purchased from New Objective (Woburn, MA). Amino acid standard was from Pierce Chemical (Rockford, IL). Bovine β-casein, sodium sulfite, sodium hydroxide, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma Chemical (St Louis, MO). Acetonitrile and trifluoroacetic acid were HPLC grade (VWR Scientific, Buffalo Grove, IL).

β-Elimination/Sulfite Addition Reaction. Various reagent concentrations shown in Table 1 were used to determine the optimum conditions for the β-elimination/sulfite addition reaction. For samples prepared for mass spectrometric analyses, 50 μL aliquots of 400 μM solutions of the model peptides KMpS, KMSpT, or KMpSpT were each incubated with a 0.1 N sodium hydroxide and 0.6 M sodium sulfite combination at room temperature, 25 °C, for 24 h. The reaction was terminated by adding 50 μL of 0.3 M acetic acid. Samples were then applied to an analytical C_{18} column for desalting using HPLC. The peptide peaks were collected and subsequently lyophilized. To determine the minimal concentration for the modification, 100−0.25 μM of KMpSpT was reacted under the same condition described above. Ten microliters of each solution was desalted by $ZipTip_{u-C18}$ according to the manufacture's manual. From 10 μL of 50% acetonitrile in 0.1% trifluoroacetic acid eluant, 1 μL was used to perform the MALDI-MS analysis.

Amino Acid Analysis. Approximately 0.1−5 μg of the dried peptides was hydrolyzed in a sealed microcapillary tube with 5.7 N constantly boiling HCl at 150 °C for 90 min, as described,[19] and analyzed with a Hitachi amino acid analyzer (model L-8800). The relative yields of cysteic acid and β-methylcysteic acid as a result of the β-elimination/sulfite addition reaction were calculated on the basis of their molar ratios relative to an amino acid standard normalized to leucine. The relative percentage of β-elimination of phosphopeptides was calculated on the basis of the decrease in serine and threonine content following hydrolysis.

Phosphorylation of Protein Kinase Substrates. A 2-nmol portion of MBP_{4-14} was phosphorylated by PKC under the conditions described by the supplier. Following the reaction, the mixture was diluted with 0.1% TFA and applied to a reversed-phase HPLC column in a gradient from 20 to 50% acetonitrile in 0.1% TFA at a flow rate of 0.5 mL/min in 30 min. Molecular weights of peptides were measured by a MALDI-TOF mass

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spectrometer, model DERP (Perspective Biosystems, Framingham, MA).

Modification of β-Casein Trypsin Digest. A 40-pmol portion of bovine β-casein was dissolved into 50 μL ammonium bicarbonate (PH 8.0) and digested with 40 ng of trypsin at 37 °C for 2 h. One microliter was removed for MALDI-MS analysis. The peptide mixture and an equal volume of 0.2 M NaOH + 1.2 M Na_2SO_3 were allowed to react for 24 h at room temperature, and the reaction was terminated by adding 50 μL of 0.3 M acidic acid. This solution was desalted by $ZipTip_{u-C18}$ according to the manufacture's manual. The eluant was pooled and freeze-dried, and the resulting peptide mixture was redissolved in 20 μL of 0.01% TFA/H_2O solution. Two microliters was used for MALDI-MS analysis, and the remaining solution was applied to the LC-coupled ion trap mass spectrometer for MS/MS analysis.

LC-ESI Tandem Mass Analysis. LC−MS/MS of the derivatized peptides was performed by a capillary HPLC (ThermoFinnigan, San Jose, CA) coupled directly to either the standard ESI source or through a flow splitter to the nanospray source utilizing a 75 μm × 6 cm fused-silica C_{18} column on the LCQ Deca mass spectrometer (ThermoFinnigan, San Jose, CA). Each peptide at 2−10 pmol based on amino acid analysis was dissolved in 0.01%TFA/0.05% acetic acid prior to column injection. A gradient from 0 to 60% consisting of 0.01% TFA/0.05% acetic acid and 0.01% TFA/0.05% acetic acid in acetonitrile was used for peptide separation. The mass spectrometer was optimized for each respective ion source with a spray voltage of 3.5−4.5 kV and a heated capillary temperature of 200 °C. Ions were isolated with a mass isolation width (m/z) set at 2.0; AGC mode on; activation Q set at 0.25; and activation time, 30 ms; and normalized collision energy was set to 35% with a default charge state set at +2. Scans were generated in both MS/MS and the Zoom scan (high resolution scan) mode of analysis. Nitrogen sheath gas was used only with the standard ESI source with flow rate set at 20.

RESULTS

Conditions for β-Elimination/Sulfite Addition of Peptides. The conversion of P-Ser and P-Thr in peptides to the corresponding sulfo-amino acids is most readily carried out in the alkali-mediated β-elimination of the phosphate group followed by sulfite addition onto the dehydro-amino acid residues (Figure 1). Published reports have indicated that sodium hydroxide at 0.5 N is the most commonly used for the β-elimination of P-Ser and P-Thr. In consideration of potential cleavage of peptide bonds, we have observed that 0.2 N NaOH at room temperature or 0.1 N NaOH at 50 °C, 24 h resulted in additional peptide peaks observed by HPLC and MALDI-TOF mass spectrometry (data not shown), suggesting that internal peptide cleavages may have occurred. In 0.1 N NaOH, similar cleavages were not detected at 25 °C and only slightly (<1%) at 37 °C. Therefore, we limited the NaOH concentration to 0.1 N throughout these studies at 25 °C and 37 °C. Table 1 shows that sulfo-amino acid formation of the two phosphopeptides, KMpS and KMSpT are both sulfite-concentration- and temperature-dependent. The yield is 85% for cysteic acid from P-Ser at 1 M Na_2SO_3, 25 °C and 90% at 0.6 M Na_2SO_3, 37 °C. P-Thr is less reactive. Only 14−40% yields were obtained from 0.1−1 M Na_2SO_3 at 25 °C; however, 74% yield was obtained at 37° in 0.1 M Na_2SO_3, and up to 85% in 1 M. Obviously, 37 °C is preferred to achieve yields comparable to those of P-Ser at 25 °C.

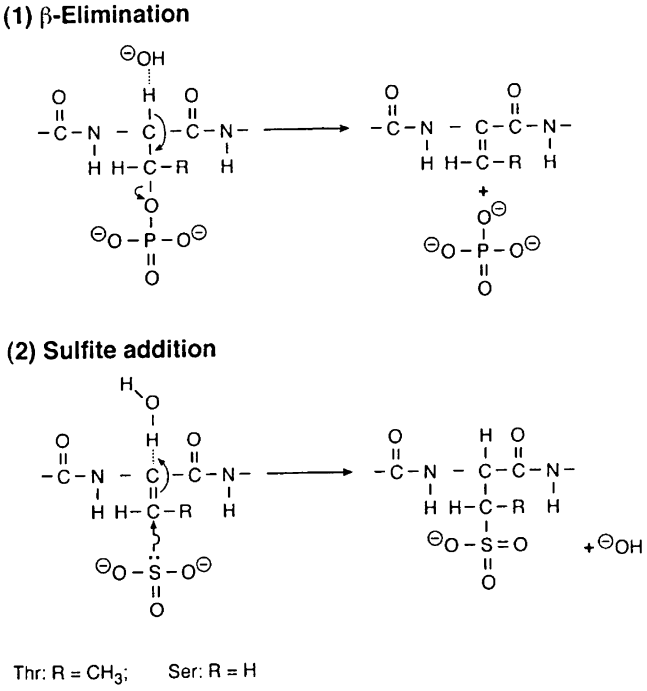


Figure 1. Mechanism for β-elimination/sulfite addition reaction.

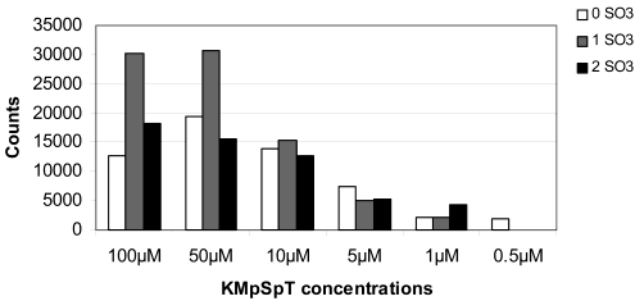


Figure 2. Yields of β-elimination/sulfite addition reaction from various concentrations of diphosphorylated peptide, KMpSpT. Human angiotensin II was used as the internal standard to normalize the MALDI-MS response in each concentration.

The difference in sulfo-amino acid formation and the disappearance of the respective phospho-amino acid may indicate the efficiency of addition. This efficiency of addition for P-Ser is 85% at 25 and 37 °C in 0.1 M Na_2SO_3, and increases to 92 and 98% at 25 and 37 °C, respectively, in 0.3 M Na_2SO_3. These data may suggest that the addition reaction in the case of P-Ser is less dependent on temperature and sulfite concentration within the range of the present study. In the case of P-Thr, this efficiency depends on the concentration of sulfite at 25 °C, as the efficiency increases from 31 to 63% with the increase of Na_2SO_3 concentrations from 0.1 to 1 M. At 37 °C, the difference of efficiency is halved between 0.1 and 1 M Na_2SO_3. Collectively, these data in Table 1 suggest that temperature plays an important role in controlling the efficiency of both β-elimination of the phosphate and addition of sulfite in Thr.

The minimally required concentration of phosphopeptide in the sulfonate conversion was also studied using diphosphorylated peptide, KMpSpT. As shown in Figure 2, the yields of both mono- and disulfonation are dose -dependent. Monosulfonation appears to be more efficient than disulfonation, and that may contribute to the low reactivity for the conversion of P-Thr, as shown in Table

1. Under the present conditions, the minimum concentration of sulfonation occurred at 1 μM. In the case of β-casein trypsin digest, a mono-sulfonated peptide was obtained at 0.8 μM concentration (see below, Figure 7).

Identification of Phosphorylation Sites in Synthetic Phosphopeptides. To minimize possible side reactions, we chose 0.6 M Na_2SO_3 in 0.1 N NaOH at 25 °C for 24 h as the condition for modification. Figures 3A and 4A show the full scan spectrum of the synthetic phosphopeptides, KMpS and KMSpT, respectively. In this mode, a loss of 98 Da (H_3PO_4) from the parent ion is noted for both peptides. These data are consistent with the observation of others when phosphopeptides are analyzed in the ESI ion trap mass spectrometer. The sulfonated peptide derived from KMpS is shown in Figure 3B at 1049.0 Da $[MH]^+$. In this spectrum, the parent ions that appeared in Figure 3A are not evident, suggesting that the modification on Ser proceeded to near completion. However, in the case of KMSpT (Figure 4B), two major ions, m/z = 1050.3 and 1065.9, are identified as the sulfonated and phosphorylated KMSpT, respectively. Clearly, significant amounts of phosphorylated peptide remained, which is expected and consistent with the results shown in Table 1.

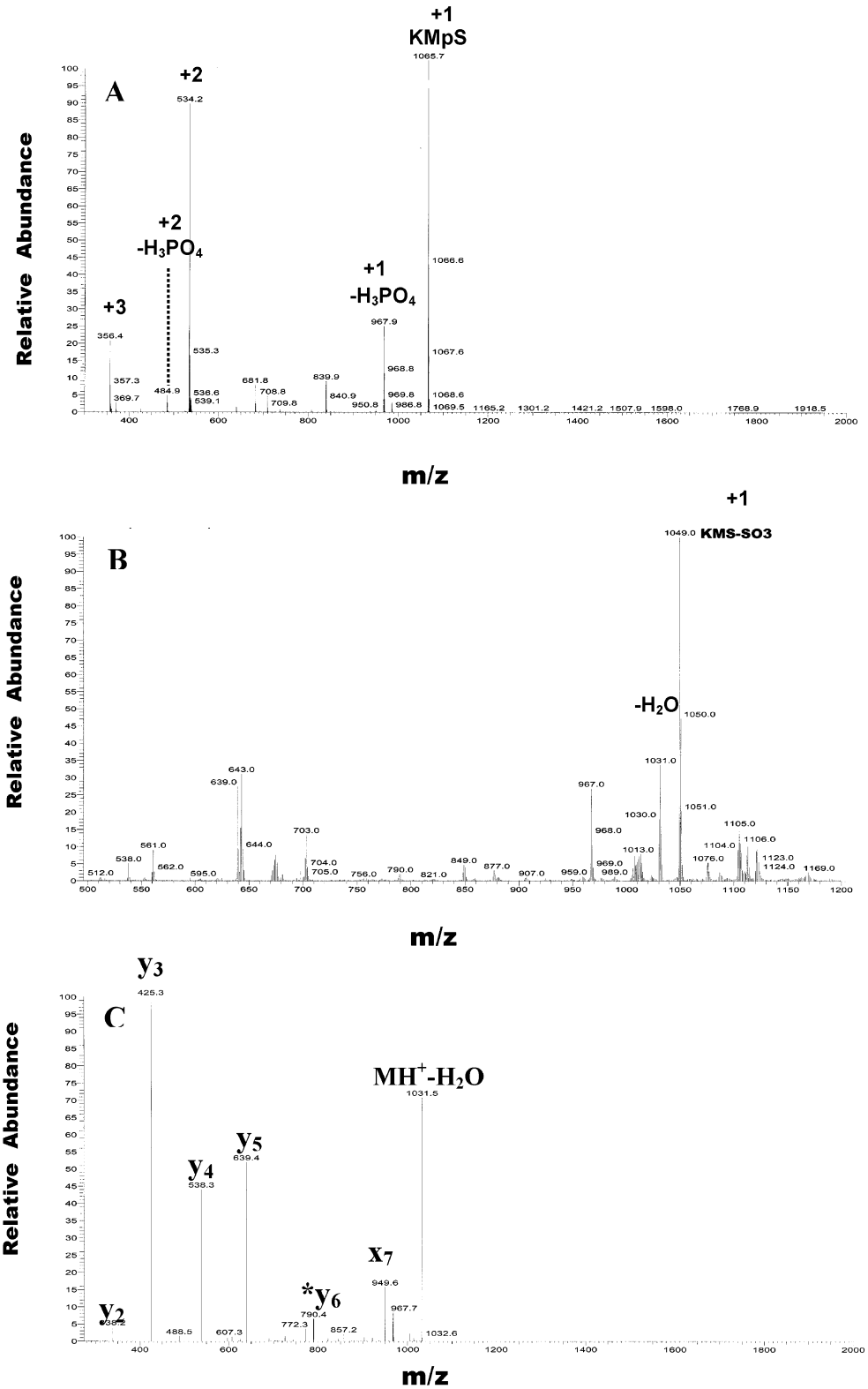
MS/MS analysis of the (KMS-SO_3 or KMsS) peptide at $[MH]^+$ = 1049.9 is shown in Figure 3C. The $*y_6$ ion (m/z = 790.4) and y_5 ion (m/z = 639.4) indicate a cysteic acid residue at the P-Ser position. No y ion corresponding to Ser at this position was detected if desulfonation had occurred. Cleavage of the alkyl carbonyl bond after the N-terminal residue Lys resulted in a product ion corresponding to x_7 (m/z = 949.6), further indicating cysteic acid at the P-Ser position. Similarly, MS/MS analysis of the doubly charged ion m/z = 526.1 (KMST-SO_3 or KMSsT, Figure 4C) indicates the series of b and y ions, which are consistent with β-methylcysteic acid at the P-Thr position of the KMSpT peptide. Furthermore, $\dagger y_5$ and $\dagger b_4$ ions were detected that confirm β-methylcysteic acid at the position of P-Thr. Again, neither y nor b ions were detected that would identify Thr in the case of desulfonation at this position.

The results of sulfonation of diphosphorylated peptide KMpSpT are shown in Figure 5. Figure 5A from the ESI full scan spectrum shows $[MH]^+$ = 1113.4, $[MH]^{2+}$ = 557.6 and is consistent with the disulfonated peptide, whereas the monosulfonated peptide was detected at $[MH]^+$ = 1129.4, $[MH]^{2+}$ = 565.5. MS/MS analysis (Figure 5B) yields nearly complete series of both b and y ions, which is therefore consistent with cysteic acid ($*b_3$ and $*y_6$) at the position of P-Ser and β-methylcysteic acid ($\dagger b_4$ and $\dagger y_5$) at the position of P-Thr.

In all three peptides above, the condition used in the present study allows unequivocal identification of the position of P-Ser and P-Thr either alone or adjacent by tandem MS/MS analysis. Furthermore, unphosphorylated Ser[6] was not modified in all cases.

Identification of Phosphorylation Site in Protein Kinase C Phosphorylated Peptide. Bovine myelin basic protein residue 4−14 peptide (MBP_{4-14}) was phosphorylated by the rat brain protein kinases C. When analyzed by reversed-phase HPLC, the phosphorylated MBP_{4-14} emerged as a major peak incompletely separated from the unreacted peptide, followed by distant PKC. MALDI-TOF analysis of the major fraction produced a major peak at m/z = 1471.3 and a minor peak of m/z = 1391.31 (data not shown). These two peaks correspond to the mass of unphosphorylated and phosphorylated MBP_{4-14}, respectively. A full-scan spectrum of the same fraction from the analysis on an LCQ Deca mass spectrometer coupled to the LC system using a 30-μm fused C_{18} column shows the phosphorylated MBP_{4-14} at $[M + H]^+$ = 1469.9, $[M + H]^{2+}$ = 735.8, $[M + H]^{3+}$ = 491.2, and $[M + H]^{4+}$ = 368.7 (Figure 6A). Another mass peak at m/z = 686.9, which is 48.9 mass unit from m/z = 735.8, may be the doubly charged dephosphorylated MBP_{4-14}, as expected because of the labile nature of the phosphate group in the ESI-ion trap. The purified phospho-MBP_{4-14} was treated using identical conditions for β-elimination/sulfite addition as performed for the synthetic peptides (Figures 3B, 4B) to obtain sulfo-MBP_{4-14}. The ESI full-scan spectrum of the sulfo-MBP_{4-14} is shown in Figure 6B. Instead of the expected mass of 1455.69, we observed an ion at m/z = 1438.3 and m/z = 720.7 as the two major mass peaks. The 17 mass difference between the expected and observed can be attributed to the loss of NH_3, suggesting that cyclization of the N-terminal Gln to form 5-oxo-L-proline, also know as pyroglutamate, may have occurred during the β-elimination/sulfite addition reaction. Figure 6C shows the MS/MS spectrum of the $[M + H]^{2+}$ = 720.7 mass peak. Identification of the fragment ion spectra yields a sequence indicating cysteic acid at position five of the consensus phosphorylation site but not Ser at position eight. Furthermore, assignment of pyroGlu at the N terminus is consistent with the major b ions found. This finding, together with those of the synthetic compounds, P-Ser in the KMpS, P-Thr in the KMSpT, and P-Ser/P-Thr in KMpSpT peptides, shows that the tandem mass sequence analysis of the sulfopeptide yields high recoveries of both b and y ion series, permitting assignments of cysteic acid and β-methylcysteic acid as the P-Ser and P-Thr, respectively, in the sequences.

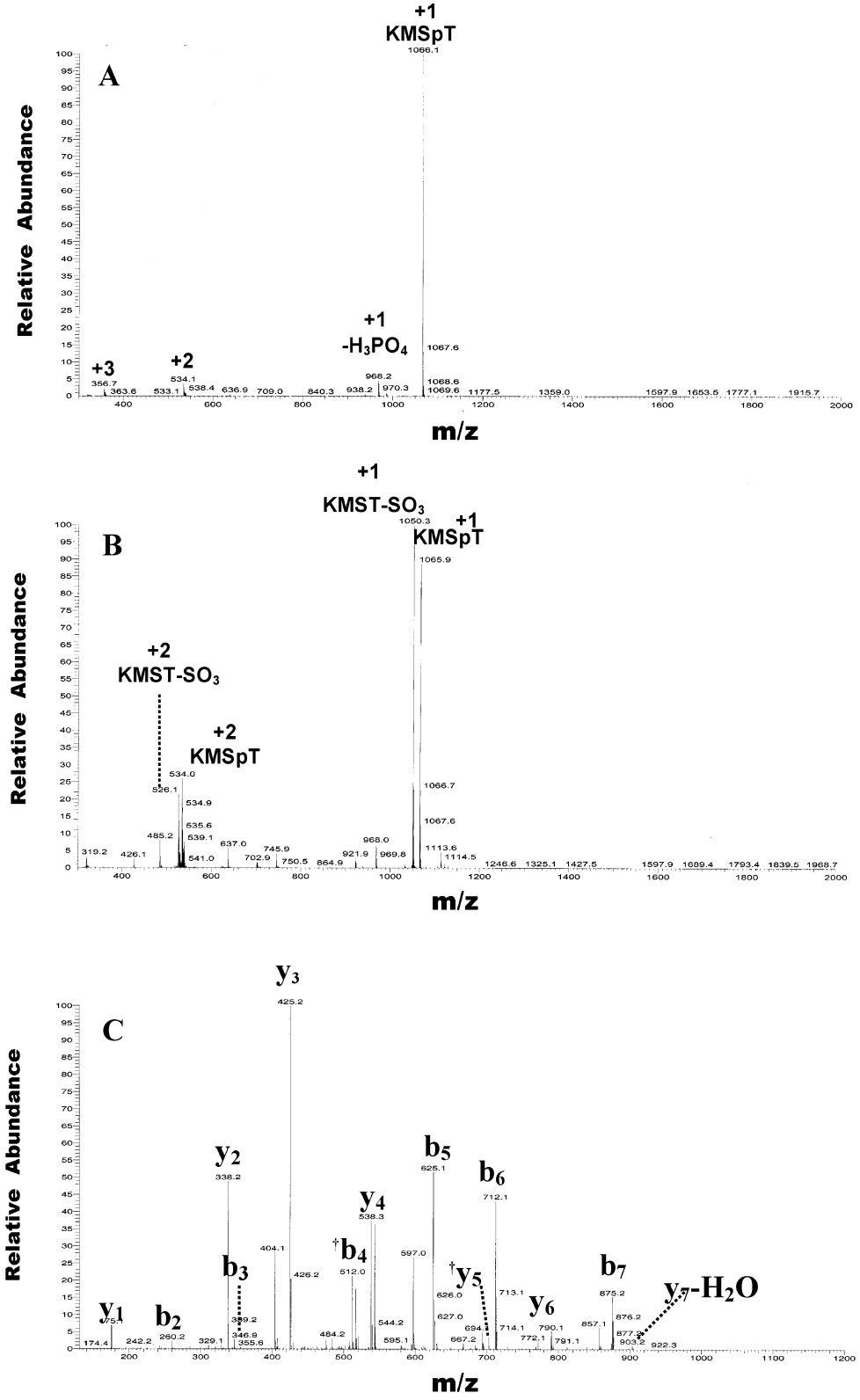
Analysis of Phosphorylation Site in β-Casein. To demonstrate the applicability of this modification method for the identification of phosphorylation site in a phosphoprotein at the picomole level, tryptic digest from 0.8 μM of β-casein was reacted in 0.1 N NaOH and 0.6 M Na_2SO_3 for 24 h at room temperature. As shown in Figure 7A, MALDI-MS analysis in the negative ion mode of the modified and desalted β-casein tryptic digest permitted the identification of the two sulfopeptides, m/z = 3059.67 (calc. 3060.31), corresponding to residues 16−40 (RELEELNVPGEIVEpSLpSpSpSEESITR), and m/z = 2046.77 (calc. 2047.09), corresponding to residues 48−63 (FQpSEEQQQTEDELQDK) of β-casein. Here, the residue numbers are based on the protein sequence deduced from the cDNA sequence of the β-casein gene. Although the monosulfonated or phosphorylated peptide could be detected in both modes, the tetrasulfonated or phosphorylated peptide could not be detected in the positive ion mode at the same peptide concentration (data not shown). It requires at least 1 order of magnitude higher concentration of the tetraphosphorylated peptide when the positive mode is used. Similarly, the full-scan MS analysis by the ESI mass spectrometer did not detect a signal for either the tetrasulfonated or the tetraphosphorylated peptide, even at more than 1 order higher peptide concentration over that used in the positive ion mode MALDI-MS analysis. However, the monosulfonated peptide was clearly detected at 1 picomole level as $[MH]^{2+}$ = 1023.8 and $[MH]^{3+}$ = 683.0 (Figure 7B). MS/MS analysis of the doubly charged ion produced nearly a complete b and y (minus y_5) ion series in which $*b_3$ (m/z = 426.7) was



Sequence	Lys	Met	CA	Thr	Leu	Ser	Tyr	Arg
Mass	128.09	131.04	151.03	101.05	113.08	87.03	163.06	156.10
y		949.6†	790.4	639.4	538.3	425.3	338.2	

†: ion observed as *x* ion.

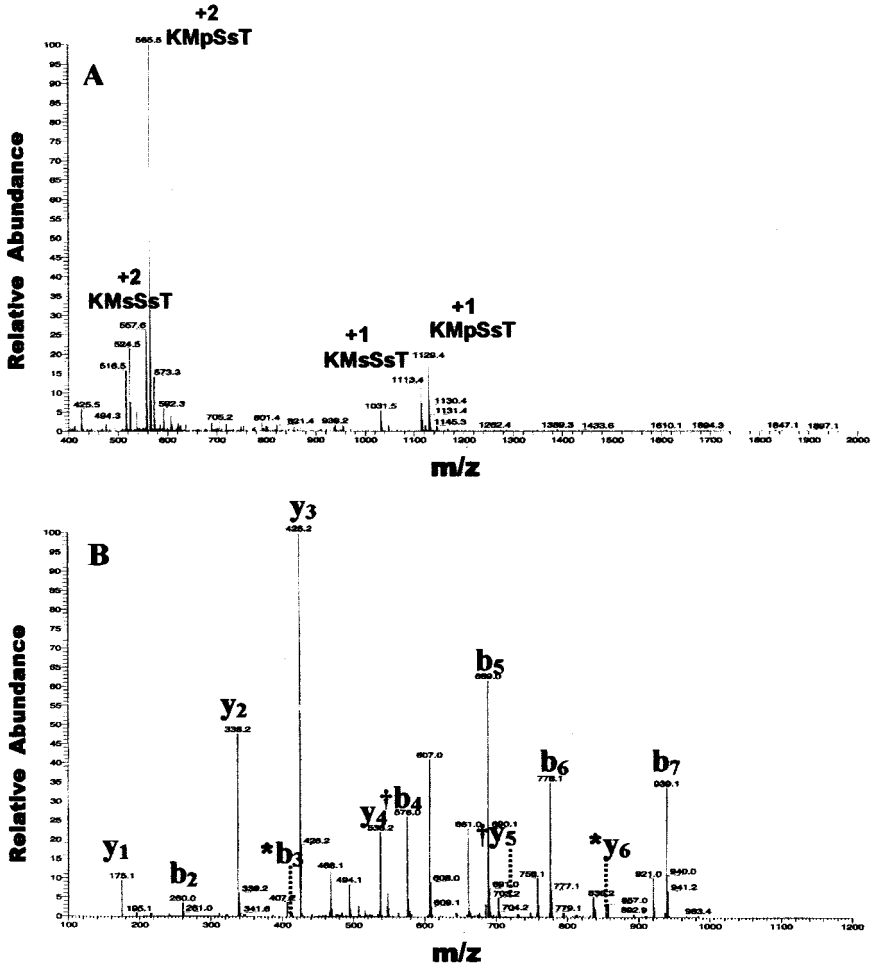
Figure 3. ESI mass spectra of phosphopeptide KMpS and sulfonated peptide KMS−SO_3 (= KMsS): (A) full-scan (MS mode only) spectrum of KMpS phosphopeptide, (B) full scan spectrum of KMsS peptide, and (C) collision-induced dissociation MS/MS spectrum of modified KMpS peptide. The ion *y denotes y ion from the cysteic acid residue.



Sequence	Lys	Met	Ser	mCA	Leu	Ser	Tyr	Arg
Mass	128.09	131.04	87.03	165.05	113.08	87.03	163.06	156.10
b		260.2	346.9	512.0	625.1	712.1	875.2	
y		903.2†	790.1	703.1	538.3	425.3	338.2	175.1

†: ion observed as *y*-H_2O ion.

Figure 4. ESI mass spectra of phosphopeptide KMSpT and sulfonated peptide KMST-SO_3 (= KMSsT): (A) full-scan spectrum of KMSpT, (B) full scan spectrum of KMSsT, and (C) collision-induced dissociation MS/MS spectrum of KMSsT. The ion †b denotes b ion, and †y denotes y ion from the β-methylcysteic acid residue.



Sequence	Lys	Met	CA	mCA	Leu	Ser	Tyr	Arg
Mass	128.09	131.04	151.03	165.05	113.08	87.03	163.06	156.10
b		260.0	411.0	576.0	689.0	776.1	939.1	
y			854.0	703.2	538.2	425.2	338.2	175.1

Figure 5. ESI mass spectra of disulfonated peptide KMsSsT: (A) full-scan spectrum of KMsSsT and (B) collision-induced dissociation MS/MS spectrum of KMsSsT. The ion *b denotes b ion, and *y denotes y ion from the cysteic acid. The ion †b denotes b ion, and †y denotes y ion from the β-methylcysteic acid residue.

identified as cysteic acid corresponding to the position of P-Ser, as shown in Figure 7C. These results further demonstrate the stability of cysteic acid derived from P-Ser for the identification of the phosphorylation site in a phosphopeptide. Furthermore, the identification is achievable for a phosphopeptide of 2 kDa modified at the low-picomole range of a protein.

DISCUSSION

Identification of specific phosphorylation sites in proteins is still recognized as being a formidable challenge for a number of reasons: the low natural abundance of signal molecules, the complexity of the peptides in the digest, and the low stoichiometry at any one phosphorylation site.[20] Techniques to identify the phosphorylation sites by direct mass spectrometry or ^{32}P-labeling coupled with Edman sequence analysis are generally thought to be difficult and not always precise. Besides, the phosphopeptide of interest must be isolated and purified first. Some methodologies are based on selective enrichment in order to elucidate the phosphorylation site(s) in a protein,[21−23] and digestion with suitable enzymes, such as trypsin, followed by separation of the phosphopeptides from the nonphosphopeptides, either on-line [24,25] or off-line,[26,27] so that only the phosphopeptides can be analyzed by mass spectrometry. Alternatively, by using the triple quadrupole mass spectrometer, phosphopeptides can be detected in the negative

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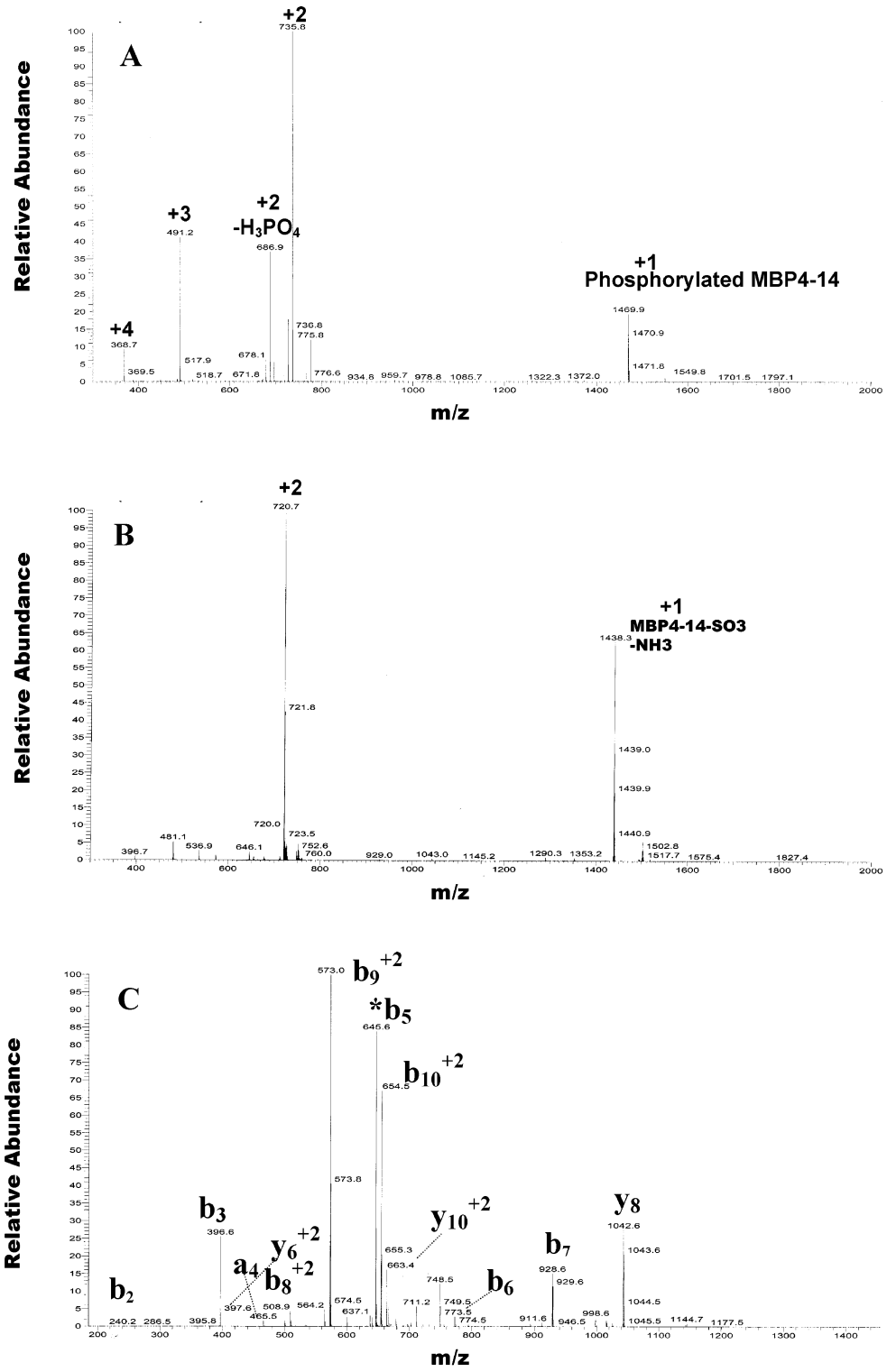

Sequence	pGlu	Lys	Arg	Pro	CA	Gln	Arg	Ser	Lys	Tyr	Leu
Mass	111.06	128.09	156.10	97.05	151.03	128.06	156.10	87.03	128.09	163.06	113.08
b		240.2	396.6	465.5[§]	645.6	773.5	928.6	508.9[†]	573.0[†]	654.5[†]	
y		663.4[†]		1042.5		397.6[†]					

[†]: observed as doubly charged ions. [§]: observed as an *a* ion. pGlu: Pyroglutamic acid residue.

Figure 6. ESI mass spectra of phosphorylated MBP_{4-14} and sulfonated MBP_{4-14}: (A) full-scan spectrum of phosphorylated MBP_{4-14} peptide, (B) full-scan spectrum of sulfonated MBP_{4-14}, and (C) collision-induced dissociation MS/MS spectrum of sulfonated MBP_{4-14}. *b denotes b ion from the cysteic acid residue.

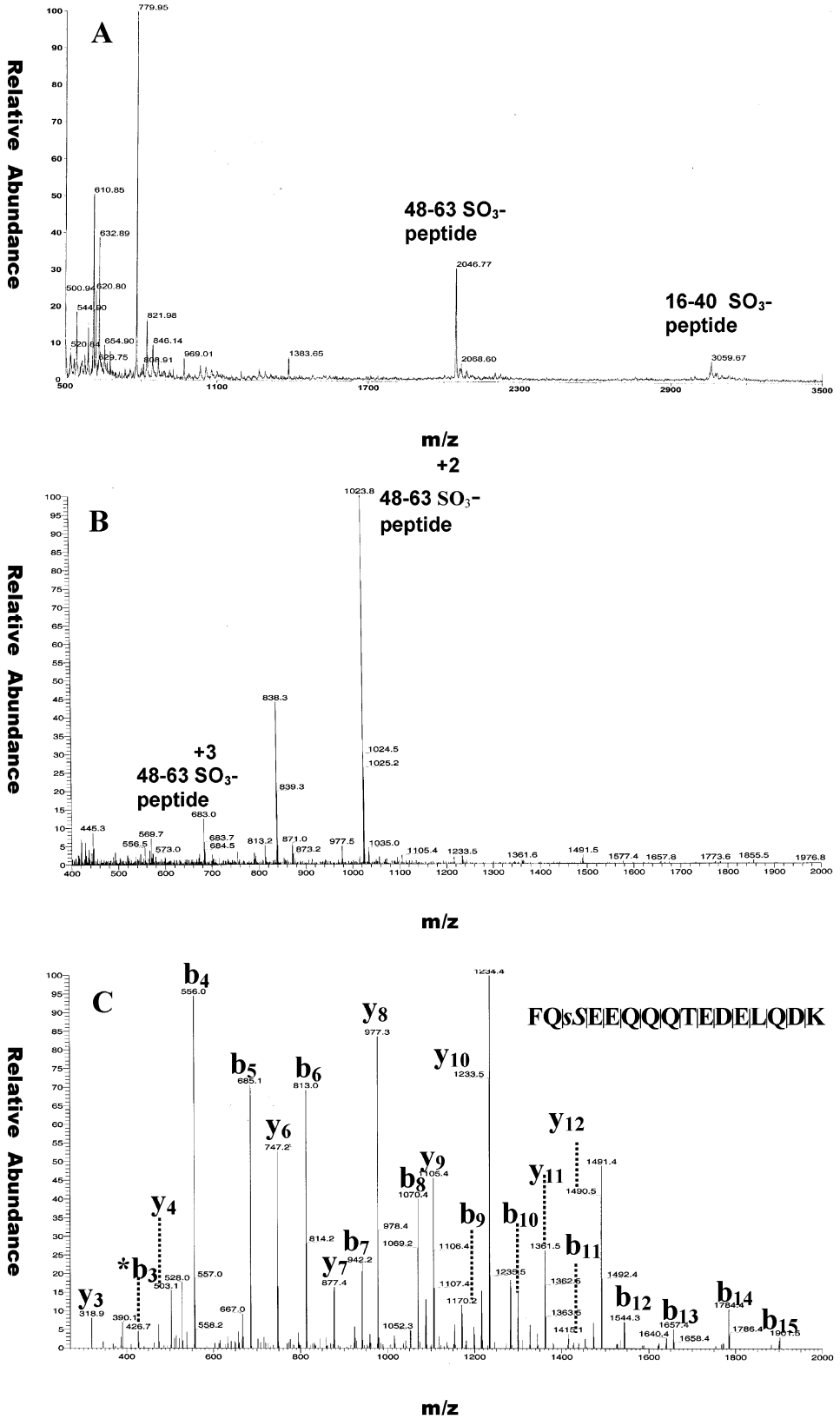

Figure 7. Mass spectra of sulfonated β-casein peptides: (A) MALDI-TOF mass spectrum of 0.1 N NaOH + 0.6 M Na_2SO_3-treated tryptic digest in negative mode, (B) full-scan spectrum of the sulfonated peptide (residues 48−63), and (C) collision-induced dissociation MS/MS spectrum of the sulfonated peptide (residues 48−63). *b denotes b ion from the cysteic acid residue.

ion mode, then sequenced in the positive ion mode.[2,4−5,28,29] Direct identification of P-Ser and P-Thr residues cannot be achieved in many cases because of the instability of the phosphate-bound linkage.[8,9] Because of the complexity of the resulting fragmentation pattern of P-Thr containing peptides,[6] methodology based on neutral loss in the tandem mass spectrometer from either P-Ser

or P-Thr is not always clear. Interpretation of the product ion spectra becomes even more complicated when there are adjacent P-Ser and P-Thr residues in the peptide, because in practice, not all fragment ions are present at detectable levels.[20]

In this study, we utilized β-elimination/sulfite addition to convert the P-Ser and P-Thr residues in the peptide into cysteic acid and β-methylcysteic acid, respectively, and tested the validity of its applicability for the identification of the phosphorylation sites in peptides by ESI quadrupole ion trap mass spectrometer. Here, we have shown that an alternative conversion of the P-Ser and P-Thr residues into compounds, which are stable during collision-induced dissociation, could produce easily interpretable product ion spectra. By incubating with 1.0 M Na_2SO_3 + 0.1 N NaOH at 37 °C for 24 h, >90% of P-Ser and 80% of P-Thr were modified in two of the model peptides, as shown in Table 1, with yields calculated on the basis of the amino acid analysis of the derivatized peptide. However, at room temperature, the conversion rate for P-Thr was somewhat slower, 40% as compared to >80% for P-Ser-containing peptide. On the basis of these results, an alternate condition utilizing 0.6 M Na_2SO_3 + 0.1 M NaOH at 25 °C was selected in the modification of phosphopeptides, thereby minimizing possible side reactions. It is well-known that P-Ser residues are resistant to β-elimination when adjacent to the amino acid proline.[11−13,30] However, when utilizing this methodology in the case of the PKC phosphorylated MBP_{4-14} peptide, the yield of the derivatized product was almost equivalent to that of the starting material. We also demonstrate the conversion of a synthetic diphosphorylated peptide, KMpSpT, as well as mono- and tetraphosphorylated peptides in bovine β-casein to their sulfonated derivatives at 1 and 0.8 μM concentrations, respectively. The clear sequence data obtained from MS/MS analyses of these mono- and diphosphorylated peptides in low-picomole levels further corroborates the advantage of the modification method described here. Although RP-HPLC-purified tetrasulfonated or tetraphosphorylated peptide was detected by the MALDI-MS in the positive ion mode using a high amount, the mass of either this tetrasulfonated or phosphorylated peptide was not observed by the ESI ion trap. This is attributable to the high acidic nature of the peptide side chain (4 SO_3^-, or 4 PO_4^{2-}, 7 γ-COO^-, but only 2 guanidino) that suppresses ESI. Previously, the other groups also encountered the same problems in analyzing this phosphopeptide.[2,24,27]

The modified peptides were identified using MALDI-TOF or ESI-ion trap mass spectrometry by observing the $[MH]^+ - 16n$ ion (n = number of modified P-Ser and P-Thr) compared to that of the phosphopeptide. In this method, side reactions are minimal as a result of the modification procedure based on analysis by HPLC and MALDI-TOF mass spectrometry. In preliminary experiments, a P-Tyr peptide was subjected to the same experimental conditions in β-elimination reaction with no change, as observed by MALDI-TOF (data not shown). Similar studies indicating the stability of P-Tyr to β-elimination have been reported by others.[15,16] In all of the examples shown, possible side reactions were noted only in the MBP_{4-14} peptide in which the N-terminal glutamine residue may have undergone cyclization to form pyroglutamic acid. This reaction, however, did not affect subsequent tandem mass analysis of the peptide. It is commonly understood that N-terminal glutamine may undergo the loss of ammonia (−17 mass) during the MS/MS fragmentation process. The derivatized product ions resulting in cysteic acid and β-methylcysteic acid residues have the advantage of being stable during the low-energy collision-induced dissociation and can be identified from either the corresponding b or y product ion series. In addition, the yields of both cysteic acid and β-methylcysteic acid can be fully determined by acid hydrolysis and subsequently measured by amino acid analysis.

It should be noted that the methodology described in this paper may not be suitable to distinguish between P-Ser and P-Thr with *O*-glycosylserine and *O*-glycosylthreonine.[18] However, these two kinds of posttranslational modifications can be differentiated by molecular mass difference using mass spectrometry, provided the sequence is known. O-Glycosylation can be removed by *O*-glycanase or other suitable means prior to modification of the peptide by β-elimination/sulfite addition.[20] A cysteine residue present in the peptide which is susceptible to β-elimination/sulfite addition must be protected by suitable means, such as alkylation, prior to modification.[17]

CONCLUSIONS

Optimal conditions for the β-elimination and sulfite addition of P-Ser and P-Thr in phosphopeptides to form cysteic acid and β-methylcysteic acid residues, respectively, are described. The stability of these sulfo-amino acid derivatives during CID allows production of sufficient y and b ion series by routine platform ESI ion trap tandem mass spectrometry and assignment of the P-Ser and P-Thr sites in peptide sequences.

Abbreviations Used: CA and mCA, cysteic acid and β-methylcysteic acid, respectively; CID, collision-induced dissociation; ESI, electrospray ionization; LC/MS, HPLC-coupled mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; PKC, protein kinases C; P-Ser, phosphoserine; P-Thr, phosphothreonine; and PTH, phenylthiohydantoin.

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