RESEARCH ARTICLE

Simultaneous Identification of Tyrosine Phosphorylation and Sulfation Sites Utilizing Tyrosine-Specific Bromination

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Abstract

Tyrosine phosphorylation and sulfation play many key roles in the cell. Isobaric phosphotyrosine and sulfotyrosine residues in peptides were determined by mass spectrometry using phosphatase or sulfatase to remove the phosphate or the sulfate group. Unique Br signature was introduced to the resulting tyrosine residues by incubation with 32% HBr at –20 °C for 20 min. MS/MS analysis of the brominated peptide enabled unambiguous determination of the phosphotyrosine and the sulfotyrosine sites. When phosphotyrosine and sulfotyrosine as well as free tyrosine were present in the same peptide, they could be determined simultaneously using either phosphatase or sulfatase following acetylation of the free tyrosine.

Key words: Phosphorylation, Sulfation, Phosphotyrosine, Sulfotyrosine, Bromination, Bromine signature

Abbreviations: pY phosphotyrosine; sY sulfotyrosine

Introduction

Post-translational tyrosine O-modifications such as tyrosine phosphorylation, O-sulfation, and O-glycosylation play critical roles in the life cycle of cells. First, tyrosine phosphorylation regulates protein's activity, localization, and interaction with other proteins in the cytosol [1–3]. Second, tyrosine sulfation modulates protein–protein interactions in the extracellular region [4–9], which is mediated by two tyrosyl protein sulfotransferases (TPST1 and TPST2)

in the trans-Golgi network [10–15], resulting in many secretory proteins and Golgi membrane-anchored proteins being sulfated on tyrosine residues [16]. Tyrosine O-glycosylation has been recently investigated in the bacterial proteins [17, 18].

Over the years, several mass spectrometric methods including enrichment and chemical modifications for the characterization of tyrosine O-modifications have been developed. However, unambiguous analysis of tyrosine O-modification by mass spectrometry (MS) still remains a challenge. Due to isobaric nature (80 Da) between phosphorylation and sulfation, it is difficult to differentiate between them without high accuracy mass measurements [19–21] or chemical modifications. Consequently, in most studies, analytical methods for tyrosine phosphorylation and sulfation have been developed separately. For tyrosine phosphorylation, immunoprecipitation-based enrichment using anti-phosphotyrosine antibody has been the standard method [22, 23]. As for the tyrosine O-sulfation, electron capture dissociation [24] and metastable atom-activated

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dissociation [25] as well as enrichment using anion exchange resin [26] have been employed. Recently, a new approach taking advantage of the labiality of the sulfate group in sulfotyrosine, easily detached under MS [27] and MS/MS conditions [27–29] has been reported by Yu et al. for analysis of tyrosine O-sulfation [30]. They converted tyrosine, both free and resulting from phosphatase treatment of phosphotyrosine, to acetyltyrosine before MS analysis so that sulfotyrosine, detected as free tyrosine due to complete neutral loss of sulfate group in the positive ion mode, could be discerned from unmodified and phosphorylated tyrosines. However, simultaneous determination of tyrosine-phosphorylation sites and tyrosine-sulfation site is still a challenge. Several methods have been developed for differentiation of phosphorylation and sulfation in natural products and carbohydrates including H/D exchange [31] and ion-pairing MS [32]. However, for simultaneous analysis of tyrosine phosphorylation (+79.9663 Da) and sulfation (+79.9568 Da) in peptides, ultra-high resolution mass spectrometer with exact mass accuracy such as FTICR or Orbitrap has been the only tool so far for differentiating these modifications based on mass difference (0.0095 Da) [21, 33].

In this paper, we propose a method for definitive characterization of post-translational tyrosine O-modifications. The ambiguity due to the neutral loss and isobaric property between tyrosine phosphorylation and sulfation is removed by selectively converting the labile group to a stable form with an isotopic tag. We developed a tyrosine-specific bromination method and applied it to selectively incorporate bromine to O-modified tyrosine residues after enzymatic hydrolysis of the O-modified tyrosine with phosphatase and sulfatase. Using this strategy, one can simultaneously determine both tyrosine phosphorylation and sulfation sites. We also show that using Br signature in the TOF/TOF analysis, one can unequivocally determine the phosphorylation or sulfation sites based on mass spectrometric detection of the brominated peptides completely eliminating the isobaric ambiguity.

Experimental

Materials and Chemicals

Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and protected amino acids used for peptide synthesis were from Novabiochem (San Diego, CA, USA). Sulfo-succinimidyl acetate (S-NHS-Ac) was from Pierce (Rockford, IL, USA). Other chemicals, enzymes, and HPLC grade solvents were from Sigma (St. Louis, MO, USA). Model peptides were synthesized by the Merrifield solid phase method. The synthesized peptides were purified by reversed-phase HPLC.

Acetylation of Tyrosine Residues

Quantitative acetylation of tyrosine residues was achieved using S-NHS-Ac with imidazole as a catalyst as described in

the recent paper with slight modification [30]. Briefly, S-NHS-Ac was added to give 30 mM concentration in 10 μ L of 1 μ M peptide mixture or BSA tryptic digest in 200 mM-N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES, pH 8.0) containing 3 mM imidazole and the mixture was incubated overnight at 4 $^{\circ}$ C; pH 8.0 was used because acetylation was complete at that pH.

Bromination of Phospho- or Sulfotyrosine Residues

Bovine serum albumin (BSA) reduced with 5 mM dithiothreitol and alkylated with 20 mM iodoacetamide was digested with trypsin (5% by wt/wt) in 25 mM ammonium bicarbonate solution for 4 h at 37 $^{\circ}$ C. Acetylation of tyrosine residues as well as amines in peptides was performed as described above. After desalting, an adequate amount of alkaline phosphatase was added to 10 μ L of the reconstituted peptide mixture in 50 mM HEPES buffer (pH 7.2) and the mixture was incubated for 30 min at room temperature. Sulfatase was used instead of phosphatase for bromination of sulfotyrosine. After clean up using ZipTip C18 (Millipore, Watford, UK), 50 μ L of the reconstituted peptide mixture in 200 mM HEPES containing 3 mM imidazole was pre-cooled to 0 $^{\circ}$ C and mixed with 100 μ L of pre-cooled (–20 $^{\circ}$ C) 48% hydrobromic acid (HBr) to give 32% HBr. The mixture was incubated at –20 $^{\circ}$ C for 20 min and desalted using PepClean C-18 spin column (Pierce, Rockford, IL, USA). Desalted sample solution was lyophilized in the speed-vac and reconstituted in 10 μ L of matrix solution for MALDI analysis. All procedures involving HBr was performed carefully in the fume hood for safety.

Mass Spectrometry

MALDI-TOF mass spectra were obtained using recrystallized α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/mL) saturated with 7 mM nitrotriacetic acid to remove CHCA clusters [34]. One μ L of the peptide solution in the matrix solution was loaded on the MTP 384 polished steel plate. Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used for MS and MS/MS measurements. Mass calibration was performed immediately before measurements using a commercial peptide mass standards kit. A typical mass spectrum was obtained by averaging 100 shots of laser. MALDI-TOF/TOF spectra were acquired by the laser-induced dissociation mode using the LIFT technique or CID mode.

Results and Discussion

Overall Strategy for Characterization of Tyrosine O-Modifications

Our strategy for characterization of tyrosine O-modifications based on tyrosine-specific bromination is illustrated in

Figure 1, assuming that a peptide has phosphorylated and sulfated tyrosine as well as free tyrosine residues. Unmodified tyrosine residues are acetylated first to prevent bromination. N-terminal and lysine amino groups are simultaneously acetylated. Then, phosphotyrosine or sulfotyrosine residues are converted to free tyrosine using alkaline phosphatase or sulfatase, respectively. Finally, Br-tag is introduced to free tyrosine residues and detected mass spectrometrically. For this strategy to work, it is important to ensure that (1) all unmodified tyrosine residues are quantitatively acetylated, (2) phosphorylated or sulfated tyrosine residues are efficiently hydrolyzed by specific enzymes, and (3) only the resulting tyrosine residues are brominated. Quantitative blocking of tyrosine residues via imidazole-catalyzed acetylation has been published [30]. Also, enzymatic selectivity and efficiency of phosphatase or sulfatase are well established.

Tyrosine-Specific Bromination

Since our strategy relies on enzymatically converting phosphotyrosine and sulfotyrosine to tyrosine and then incorporating Br signature to the resulting tyrosine residue, it is important to ensure that only tyrosine, not acetyltyrosine, phosphotyrosine, or sulfotyrosine becomes brominated. To demonstrate tyrosine-specific bromination, a mixture of six synthetic tryptic peptides containing sulfated, acetylated, or phosphorylated tyrosine as well as unmodified tyrosine residues (EVsYR, aLNlaYR, AEGVDYR, AVTpYLQR, AVYTLpYR, LGYDpYEYAR; *a*, *p*, and *s* stand for acetylation, phosphorylation, and sulfation, respectively) at 1 μ M concentration each was subjected to the optimized bromination condition. As shown in Figure 2, the sulfotyrosine-containing peptide (EVsYR at 644.45 in the negative ion mode,

but at 566.40 in the positive ion mode due to complete neutral loss of the sulfate group), the acetyltyrosine-containing peptide (aLNlaYR at 762.4), and the phosphotyrosine-containing peptide (AVTpYLQR at 930.45), all without unmodified tyrosine, were unchanged before (Figure 2a) and after (Figure 2b) bromination reaction. On the other hand, AEGVDYR and AVYTLpYR containing one unmodified tyrosine showed 78 D shift, from 809.38 and 965.45 to 887.29 and 1043.36, respectively, corresponding to single bromination. Moreover, a unique Br signature was clearly observed. LGYDpYEYAR with two tyrosine residues showed 156 D shift, from 1229.49 to 1385.31, representing double bromination with another unique di-Br signature. The mass spectrum in Figure 2b also shows that the bromination reaction is nearly quantitative. TOF/TOF analysis of the brominated peptides confirmed the specificity of bromination on the unmodified tyrosine (Supplemental Figure 1).

It should be noted that even though no bromination peak was observed from peptides containing acetylated-, phosphorylated-, and sulfated tyrosine when the reaction was carried out at -20°C , at room temperature, acetylated and sulfated, but not phosphorylated, tyrosine residues were partially brominated after hydrolysis of its O-modified groups (Supplemental Figures 2b and 3) [35, 36]. Even at 0°C , some brominated peaks were observed from acetylated and sulfated tyrosine residues after 10 min incubation (Supplemental Figure 2c). After 1 h at room temperature, tyrosine was doubly brominated (Supplemental Figure 4). These results show that even sulfotyrosine labile at low pH is stable and not brominated at -20°C . Thus, any sulfotyrosine or phosphotyrosine remaining from incomplete enzymatic hydrolysis would not interfere in the analysis of the brominated tyrosine derived from sulfotyrosine or

Figure 1. Strategy for bromination of phosphotyrosine and sulfotyrosine residue. Free tyrosine and primary amine are acetylated quantitatively using S-NHS-Ac/imidazole. Then phosphotyrosine and sulfotyrosine are separately hydrolyzed by alkaline phosphatase and sulfatase, respectively. Resulting free tyrosine is brominated by incubation in HBr. Free tyrosine (Y), phosphotyrosine (pY), and sulfotyrosine (sY) were detected as acetyltyrosine (aY), bromotyrosine (BrY), and tyrosine (Y), respectively, when phosphatase was used, while they were detected as aY, pY, and BrY, respectively, when sulfatase was used

Figure 2. MALDI-TOF mass spectra from a mixture of six peptides (a) before and (b) after bromination reaction showing selective bromination of free tyrosine residue

phosphotyrosine. It is important, however, that free tyrosine residues are completely acetylated beforehand to prevent bromination.

This specific tyrosine bromination can be used in conjunction with another highly specific, enzymatic process to identify phosphotyrosine or sulfotyrosine residues. If phosphatase is used, phosphotyrosine would be hydrolyzed and detected as bromotyrosine upon bromination while sulfotyrosine would be detected as unmodified tyrosine in the positive ion mode after loss of the sulfate group. Tyrosine unmodified in the original peptide would be detected as acetyltyrosine after the initial acetylation reaction. On the other hand, if sulfatase is used, sulfotyrosine and unmodified tyrosine would be detected as bromotyrosine and acetyltyrosine, respectively, while phosphotyrosine is unchanged. Thus phosphotyrosine, sulfotyrosine, and free tyrosine could be distinguished from each other using either phosphatase or sulfatase. Even endogenous bromotyrosine existing at very low concentration in biological samples [37, 38] could be distinguished from brominated tyrosine derived from O-modified tyrosine because endogenous bromotyrosine should be acetylated and show different mass shift from brominated tyrosine (Supplemental Figure 5).

Determination of the Phosphorylation Site

First we used phosphatase to determine phosphotyrosine site. Figure 3a shows the MALDI mass spectra obtained in

the negative and positive ion mode from a mixture of seven peptides containing tyrosine, phosphotyrosine, or sulfotyrosine residues (EVsYR, AEGVDYR, AVYTLpYR, LNIYR at 678.39, LpYGDYAR at 937.38, EGANpYDV sYSR at 1253.48 (1331.43 in the negative ion mode), DGVpYYVpYYER at 1486.54). Figure 3b shows that all N-terminal amines and tyrosine residues, but not phosphorylated and sulfated tyrosine or other residues, are quantitatively acetylated in the presence of imidazole catalyst [30]. After phosphatase treatment, all phosphopeptides showed loss of 80 Da per phosphate group (Figure 3c). Upon bromination, the 941.44, 969.51, and 1215.53 peaks were shifted by 78 Da and showed a unique mono-Br signature from one Br atom (1019.35, 1047.42, and 1293.44 in Figure 3d). The 1452.63 peak was shifted by 156 Da and showed another unique di-Br signature from two Br atoms (1608.45 in Figure 3d). Thus, when phosphatase was used, only phosphotyrosine residue ended up carrying the Br signature making its selection easy for further MS/MS analysis.

All four brominated peptides in Figure 3d were subjected to TOF/TOF analyses. Figure 4a shows a series of y-ions from LpYGDYAR consistent with the sequence and the phosphorylation site. The parent MH^+ peak at 1019.35 carries the mono-Br signature. The mass difference between the parent and the y6 peak is consistent with the N-terminal acetyl leucine/isoleucine. The y6 peak carries the Br signature, but not the y5-y1 peaks, which clearly shows that the second residue from the N-terminus is phosphotyrosine. The mass difference of 241 Da between y5 and y6 is consistent with bromotyrosine derived from phosphotyrosine.

Figure 3. Selective bromination of phosphotyrosine residues in a peptide mixture. **(a)** MALDI-TOF mass spectrum from a mixture of seven peptides in both negative and positive ion mode. Two peptides with sulfotyrosine (*) showed 78 Da mass shift between the negative mode and the positive mode (from 644.24 to 566.29 and from 1331.43 to 1253.48) due to loss of the sulfate in the positive ion mode. **(b)** Same peptides after acetylation showing completion of the reaction. **(c)** Acetylated peptides after treatment with alkaline phosphatase. **(d)** After bromination with HBr. The bromine signature indicates the number of phosphotyrosine residues

The mass difference of 205 Da between y2 and y3 corresponds to acetyltyrosine and shows that the fifth residue from the N-terminus was unmodified tyrosine. The b-series ions carrying the Br signature also confirmed the sequence and the phosphotyrosine site. The mass difference between b1 and b2 was again 241 Da the same as the y5-y6 difference. The other mono-brominated peptide, AVYTLpYR at 1047.42 in Figure 3d, was similarly analyzed and their

phosphorylation site also clearly identified (Supplemental Figure 6).

When there were two phosphotyrosine residues in the original peptide leading to two bromotyrosines (Figure 3d), the Br signature again proved to be quite useful. In Figure 4b from DGVpYYVpYYER, the MH^+ ion with the di-Br signature at 1608.45 indicates that there are two phosphorylation sites in the peptide. The y1-y3 ions were

Figure 4. Identification of tyrosine phosphorylation site. MALDI-TOF/TOF spectra of the brominated $LpYGDYAR$ (a) and $DGVpYVpYER$ (b) in Figure 3d

without the Br signature, y4–y6 ions with the mono-Br signature, and y7, y9, and MH^+ with the di-Br signature in the same MS/MS spectrum. The mass difference as well as the change of isotopic signature between y3 and y4, and y6 and y7 indicates that both the fourth and the seventh residues are bromotyrosine derived from phosphotyrosine. The originally unmodified tyrosine residues (fifth and eighth) were observed as acetyltyrosine from the mass difference between y2 and y3, and y5 and y6. Similarly, b-ion series partially detected confirm that the fourth residue is bromotyrosine. Thus, we were able to identify two phosphotyrosine sites in a peptide containing a total of four tyrosine residues without any ambiguity. These results demonstrate that the Br signature introduced following phosphatase treatment pinpoints which tyrosine residue is post-translationally phosphorylated in a peptide containing both modified and unmodified tyrosine.

Simultaneous Determination of Phosphorylation and Sulfation Sites

The two isobaric O-modified tyrosines in $EGANpYDV_sYSR$ peptide containing both phosphotyrosine and sulfotyrosine were characterized using either phosphatase or sulfatase as outlined in Figure 1. The $EGANpYDV_sYSR$ peptide treated with phosphatase and brominated approach is shown at m/z 1293.44 in Figure 3d. In the TOF/TOF analysis of the 1293.44 ion, five y-ions from the MH^+ ion to y6 ion at 880.28 were observed with the mono-Br signature (Figure 5a). The mass differences were consistent with the EGAN sequence. y5 ion is without the Br signature and the mass difference of 141 Da between y5 and y6 clearly shows that the next residue after EGAN in the original peptide is phosphotyrosine. Further sequence could be determined from the y4–y1 ions. The sulfotyrosine residue was

Figure 5. MALDI-TOF/TOF spectra of brominated EGANpYDV_sYSR after phosphatase treatment (a) or sulfatase treatment (b)

identified from y2 and y3 ions. The mass difference of 163 Da between y2 and y3 ion corresponds to tyrosine resulting from 80 Da loss from sulfotyrosine during the MS/MS process. Unmodified tyrosine in the original peptide would have been converted to acetyltyrosine. Thus, 163 Da difference in the MS/MS spectrum is an indirect evidence of sulfotyrosine. This example demonstrates that phosphotyrosine and sulfotyrosine are detected as bromotyrosine and tyrosine, respectively, and can be determined simultaneously using phosphatase and bromination reactions.

Sulfatase can be used for a more direct determination of sulfotyrosine in EGANpYDV_sYSR incorporating the Br signature to the sulfotyrosine residue. In this case, the acetylation step was skipped because the peptide has no unmodified tyrosine and there is no need to distinguish

between the free tyrosine and the sulfotyrosine-derived tyrosine. After bromination following sulfatase treatment, the brominated peak (m/z 1331.39) was subjected to MS/MS analysis; y1 and y2 peaks are without the Br signature, and all subsequent y ion peaks show the Br signature (Figure 5b). Moreover, the mass difference of 241 Da between y3 and y2 peaks conclusively shows that the eighth residue is sulfotyrosine. The mass difference of 243 Da between y5 and y6 peaks is consistent with phosphotyrosine. The isobaric tyrosine phosphorylation was unchanged by sulfatase and bromination procedure. The b1–b7 ions were without the Br signature as expected. Unfortunately, the b8 ion with the signature was not detected. This result shows that both sulfotyrosine and phosphotyrosine can be determined simultaneously by sulfatase treatment.

Figure 6. Identification of tyrosine phosphorylation site in *LpYGDYAR* in the presence of 10-fold molar excess of bovine serum albumin (BSA) tryptic peptides. (a) Mass spectrum of BSA digest after acetylation. All peptides are fully acetylated. *LpYGDYAR* is low in intensity. (b) After dephosphorylation and bromination, the phosphopeptide at 1019.35 shows bromine signature. Amino acid residues and the number of acetylation are indicated for BSA tryptic peptides detected

Limitations of the Br Signature

The Br signature as an isotopic tag has been used for peptide sequence analysis in several studies [39–42]. Yet, it has limitations in terms of the mass range of peptides and the number of Br atoms [42]. The Br signature in mono-brominated peptide should be most evident for peptides up to around 1500 Da. For peptides over 2500 Da, the doublet shape of mono-brominated peptide completely disappears as the second isotopic peak originating from ^{13}C becomes stronger than the monoisotopic peak. Di-Br signature has a similar limitation. Also, the di-Br signature in high molecular weight peptides (>2000 Da) is rather complicated and of limited use. However, the Br signature might be recognized in the MS/MS spectrum even for peptides where it is difficult to detect the signature in the MS spectrum. Practically, mono- or di-Br signature in peptides within ~2000 Da would be most useful in both MS and MS/MS.

Potential Application to Real Samples

Post-translationally modified peptides are usually present at low concentrations in real samples unless highly purified. Therefore, it is important to show that the method is applicable when excessive amounts of other peptides are present. One μM BSA tryptic digest was mixed with *LpYGDYAR* at 0.1 μM concentration. The phosphopeptide peak was not even visible in the mass spectrum (Supplemental Figure 7a). After acetylation, a small peak corresponding to

di-acetylation was observed at 1021.40 (Figure 6a). The dephosphorylated peptide peak at 941.44 became stronger (Supplemental Figure 7b). After bromination, a small peak readily identified by the Br signature was observed at 1019.35 (Figure 6b). This peak could be used to get the MS/MS spectrum and the sequence information shown in Figure 4a. It appears that the modification via phosphatase and bromination steps has a dual benefit of introducing the Br signature and removing the negatively charged phosphate group enhancing the MALDI signal intensity of the phosphotyrosine-containing peptide.

The tyrosine O-glycosylation recently found in bacteria proteome [17, 18] may be another target for specific bromination. Following glycosylase treatment and bromination, tyrosine O-glycosylation site could be determined with Br signature. Our method is expected to be applicable for all tyrosine O-modifications discovered so far.

Conclusion

Br-tag can be incorporated specifically to tyrosine residues generated from phosphatase or sulfatase treatment of phosphotyrosine or sulfotyrosine residues following acetylation of the original tyrosine. The phosphotyrosine or sulfotyrosine sites can be determined unequivocally utilizing the Br signature. When phosphotyrosine and sulfotyrosine as well as free tyrosine are present in the same peptide or a peptide mixture, they can be determined simultaneously using either phosphatase or sulfatase following acetylation

of the free tyrosine. The method could be used in other types of tyrosine O-modifications.

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