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Analysis of Phosphorylated Peptides by Double Pseudoneutral Loss Extraction Coupled with Derivatization Using *N*-(4-Bromobenzoyl)aminoethanethiol

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The analysis of post-translational phosphorylation is a crucial step in understanding the mechanisms of many physiological events. Numerous approaches for the development of analytical methods aimed at the detection and quantification of phosphorylated proteins by mass spectrometry have been reported in the literature. In this paper, we report a new strategy for the identification of phosphorylated serine and threonine residues in phosphoproteins. This method consists of selective derivatization of phosphoproteins coupled with double pseudoneutral loss extraction after nanoLC/ESI-MS/MS analysis. First, we designed and synthesized a new derivatization reagent, *N*-(4-bromobenzoyl)aminoethanethiol, which can selectively react with α,β-unsaturated ketones produced by β-elimination of a phosphoryl group from phosphorylated serine or threonine residues. The mass spectrum of the derivatized peptide shows a product ion with a characteristic isotopic pattern. After derivatization, fragment ions of peptides with phosphoserine or phosphothreonine have twin peaks with an intensity ratio of approximately 1:1 and a difference of two mass units, while product ions from peptides without phosphoserine or phosphothreonine have normal isotopic patterns. Therefore, the neutral loss of the derivatized residue in the product ion mass spectrum includes two difference losses caused by ^{79}Br and ^{81}Br. The extraction of the product scan mass spectrum with double pseudoneutral losses is very effective for identification of the derivatized peptides produced from phosphorylated peptides. The new strategy represents a useful tool for the analysis of phosphorylated serine and threonine residues in phosphorylated proteins.

Post-translational phosphorylation of proteins regulates many physiological events, such as cell growth, proliferation, differentiation, and apoptosis.[1] Reversible phosphorylation and dephosphorylation are strictly controlled by the activity modulation of kinase and phosphatase.[2] To understand the molecular mechanisms of the physiological events regulated by post-translational phosphorylation, a reliable method for the analysis of phosphorylation of proteins is required. The detection of phosphorylated peptides in a complex peptide mixture is generally difficult because of the low concentration of phosphorylated peptides in the mixture. The selective concentration of phosphorylated peptides is one useful approach to overcoming this problem. An antiphosphorylated tyrosine antibody has been used for affinity extraction of phosphorylated tyrosine-containing peptide fragments in complicated peptides,[3,4] but the generation of high-quality antibodies with sufficient affinity and selectivity for phosphorylated serine- and threonine-containing peptides has been difficult. In immobilized metal affinity chromatography, bound metal ions on a chelating support capture phosphoryl groups of phosphopeptides by complexation,[5−7] but the process is not completely selective, and the undesirable complexation of acidic amino acid-containing peptides frequently occurs as well.[8] Ishihama et al. have reported an effective method for phosphopeptide enrichment using metal oxide chromatography combined with aliphatic hydroxy acids, in which the added hydroxy acids enhance selectivity by preventing the incorporation of acidic amino acid-containing peptides.[9] On the other hand, Oda et al. developed a process involving the Michael addition of biotin to α,β-unsaturated ketones derived from the β-elimination of phosphoryl groups in phosphorylated resi-

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dues.[10] The biotin-modified peptides were then selectively isolated using the avidin−biotin binding property.

Mass spectrometry (MS) is a powerful tool useful for analyzing phosphorylated peptides. Because phosphorylated peptides possess strong negative charges and therefore have a low ionization efficiency in the positive ion detection mode on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS),[11] postsource decay has been used for the identification of phosphorylated amino acid residues.[12,13] In electrospray ionization (ESI)-MS, common positive ion detection analysis can detect phosphorylated peptide ions as well as other nonphosphorylated peptide ions. However, the elimination of H_3PO_4 from phosphorylated peptides is more frequent than backbone cleavages on the low-energy collision-induced dissociation (CID), making the analysis of phosphorylated peptides difficult with ESI-MS.[14] Precursor ion scan analysis can effectively identify the precursor ion of a phosphorylated peptide by monitoring the PO_3^- fragment at m/z 79 in the negative ion detection mode, and the process is facilitated by the high sensitivity to PO_3^- caused by lower background levels under low-pH conditions.[15] In the positive ion detection mode, the method for the detection of the phosphotyrosine immonium ion at m/z 216 has been used to easily identify phosphotyrosine-containing peptides,[16] and a neutral loss scan of H_3PO_4 has also been used to detect a phosphorylated peptide with CID.[17]

Because MS is effective in the separation of isotopes from each other, a characteristic isotopic pattern facilitates the selective identification of target compounds. For example, when proteins are enzymatically digested in a 1:1 mixture of $H_2{}^{18}O$ and $H_2{}^{16}O$, the ^{18}O atom is introduced into the C-terminus with a 50% probability.[18] The C-terminus peptides are easily distinguishable from other peptides because only the C-terminus from the precursor protein have natural isotopic patterns.[19] Bromine has two natural nuclear species with mass numbers of 79 and 81. Therefore, a compound containing one bromine atom has a characteristic isotopic pattern on MS of two peaks with almost the same intensity and a difference of two mass units. Taking advantage of bromine's signature MS pattern, Miyagi et al. developed a straightforward amino acid sequence analysis method in which the amino termini of peptides were derivatized using *N*-bromonicotinic acid *N*-hydroxysuccinimidyl ester.[20] We proposed the application of the signature MS pattern of bromine for the identification of phosphorylated peptides. In this paper, we developed a new derivatization reagent, *N*-(4-bromobenzoyl)aminoethanethiol, which includes one bromine atom and a thiol group, and its structure and synthesis were shown in Figure 1A. After Michael addition of the thiol group to α,β-unsaturated ketones (**6**) produced by the β-elimination of a phosphoryl group from phosphoserine and phosphothreonine (**5**) (Figure 1B), the bromine-containing thioether products (**7**) are easily detected by MS. This approach represents a useful analytical method for the detection of phosphorylated proteins.

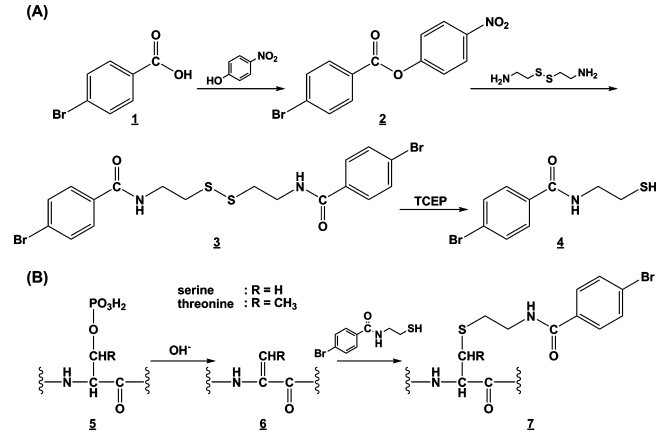

Figure 1. Synthesis of *N*-(4-bromobenzoyl)aminoethanethiol (A) and derivatization of phosphoserine and phosphothreonine residues (B).

EXPERIMENTAL SECTION

Reagents and Apparatus. α-Cyano-4-hydroxycinnamic acid (CHCA), the matrix for MALDI-TOF/MS, was supplied by Aldrich Chemical Co. (Milwaukee, WI). Sequence-grade modified trypsin was obtained from Promega (Madison, WI). Dithiothreitol, iodoacetamide, cystamine hydrochloride, *p*-bromobenzoic acid, and *p*-nitrophenol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). β-Casein, bovine serum albumin, immunoglobulin G, α-chymotrypsinogen A, catalase, carbonic anhydrase, transferrin, plasmin, myoglobin, and lysozyme were purchased from Sigma-Aldrich Inc. (St. Louis, MO). A model phosphorylated peptide (FAGSSYApSFK) was synthesized by Fmoc solid-phase chemical synthesis using 4-hydroxymethylphenoxymethyl-*co*-polystyrene-1% divinylbenzene resin. ZipTip C_{18} cartridges were purchased from Millipore (Milford, MA). Ultrapure water was prepared using a PURELAB ultra-apparatus (Organo Co., Ltd., Tokyo, Japan). All other reagents were analytical grade and solvents were HPLC or LC/MS grade.

MALDI-TOF/MS was performed using a Voyager DE-STR spectrometer (Applied Biosystems, Framingham, MA) equipped with an N_2 laser (337 nm). In positive ion mode, a matrix solution of fresh saturated CHCA in water/acetonitrile/trifluoroacetic acid (TFA) (50:50:0.1, v/v/v) was used. Peptide samples dissolved in 0.5 μL of water/acetonitrile/TFA (25:75:0.1, v/v/v) were mixed with 0.5 μL of the matrix solution on the MALDI plate. Spectra were calibrated internally using the monoisotopic protonated molecules of two peptide standards, angiotensin I and an adrenocorticotropic hormone 7−38 (American Peptide Co., Inc., Sunnyvale, CA), in the matrix solution.

An HPLC-isolated fraction of a derivatized peptide in 100 μL of water/methanol/formic acid (50:50:1, v/v/v) was infused at a

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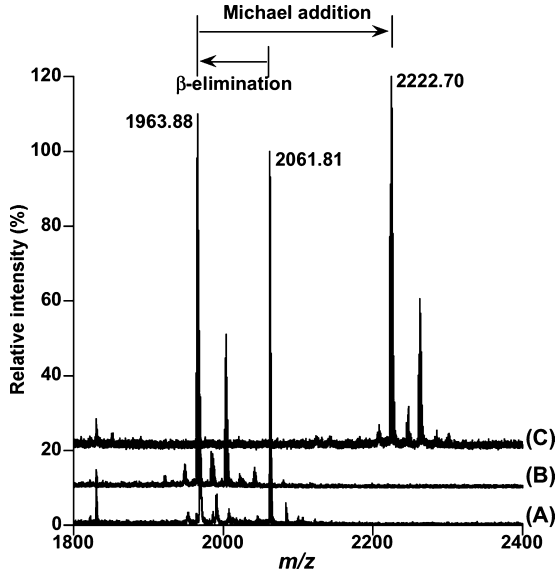

Figure 2. MALDI-TOF mass spectra of a phosphorylated peptide fragment (amino acids 33−48) from β-casein (A), its β-eliminated intermediate (B), and the final derivatized product (C). MS conditions: instrument, Voyager DE-STR (reflector mode); matrix, CHCA; accelerating voltage, 25 kV; grid voltage, 18 kV; guide wire voltage, 50 V.

flow rate of 200 nL/min into a $QSTAR_{XL}$ mass spectrometer (Applied Biosystems) equipped with a handmade nanoESI probe attached to a metal nanosprayer (GL Sciences, Tokyo, Japan). The product ion scan was performed utilizing an ionspray voltage of 3000 V, a declustering potential of 60 V, a collision energy of 50 eV, and a collision gas (nitrogen) of 3 units.

Nanoscale-liquid chromatography/electrospray ionization tandem mass spectrometry (nanoLC/ESI-MS/MS) was performed using a NanoFrontier LD (Hitachi High-Technologies Co., Tokyo) equipped with NanoFrontier nLC (Hitachi High-Technologies Co.). The prepared samples were injected into the system with an injection volume of 1.0 μL, and peptides were concentrated for 3 min (10 μL/min) on a MONOLITH TRAP C18 trapping column (25 mm length × 50 μm I.D., Hitachi High-Technologies) using water/methanol/acetic acid (98:2:0.1, v/v/v). The peptides were transferred from the trapping column to the analytical column, a PicoFrit C18 column (100 mm length × 75 μm i.d., New Objective, Woburn, MA) by switching the flow channel. The separation was performed at a flow rate of 200 nL/min using a linear gradient elution method of 5%−80% mobile phase B over 60 min: mobile phase A, water/methanol/formic acid (98:2:0.1, v/v/v); mobile phase B, water/methanol/formic acid (2:98:0.1, v/v/v). NanoFrontier nLC was controlled by NanoLC Control Panel (ver. 1.0.0.1) software, and NanoFrontier LD was controlled by Control Panel (ver. 1.0.0.0) software. Those two types of software worked under the control of Auto Sequence (ver. 0.0.0.1) software. The measurements were performed by IBA (information based acquisition) mode. The spray potential, Ex potential, and AP2 potential were 2000 V, 105 V, and 45 V, respectively. Both AP1 and AP2 temperatures were 140 °C, and the flow rate of curtain gas (nitrogen) was 0.8 L/min. Spectrum acquisition settings were as follows: scan range, 100−2000 amu; microsequence, 81; accumulation time, 20.0 ms; MS/MS isolation time, 10.0 ms; MS/MS isolation width, ±5 amu; CID time, 10.0 ms; CID gain, auto. The software chose up to three target peaks for MS/MS scan by MS scan. Data analysis was performed by using NanoFrontier LD Data Processing (ver. 1.0.0.0), which was specially modified to equip double pseudoneutral loss extraction function.

Synthesis of *N*-(4-Bromobenzoyl)aminoethanethiol. The synthesis route is shown in Figure 1A. To a solution of *p*-bromobenzoic acid (**1**, 500 mg, 2.5 mmol) in dioxane (10 mL) were added 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (500 mg, 2.6 mmol) and *p*-nitrophenol (300 mg, 2.2 mmol). The reaction mixture was stirred at room temperature overnight and then evaporated in vacuo. Ethyl acetate (300 mL) and saturated aqueous sodium hydrogen carbonate (100 mL) were added to the resultant residue, and the organic layer was evaporated in vacuo to afford *p*-bromobenzoic acid *p*-nitrophenyl ester (**2**).

A solution of the obtained activated ester (**2**) in tetrahydrofuran (50 mL) was added to a solution of cystamine dihydrochloride (480 mg, 2.1 mmol) in water/tetrahydrofuran/triethylamine (60 mL, 40:20:1.2, v/v/v). After stirring at room temperature for 1 h, the reaction mixture was concentrated in vacuo to remove the organic solvent, and ethyl acetate (250 mL) was added. The organic layer was washed with 5% aqueous hydrogen chloride (3 × 100 mL) to remove unreacted cystamine and then concentrated in vacuo. The residue was dissolved in a mixture of tetrahydrofuran (50 mL) and 1% aqueous sodium hydroxide (50 mL) for hydrolysis of unreacted *p*-nitrophenyl ester and stirred at room

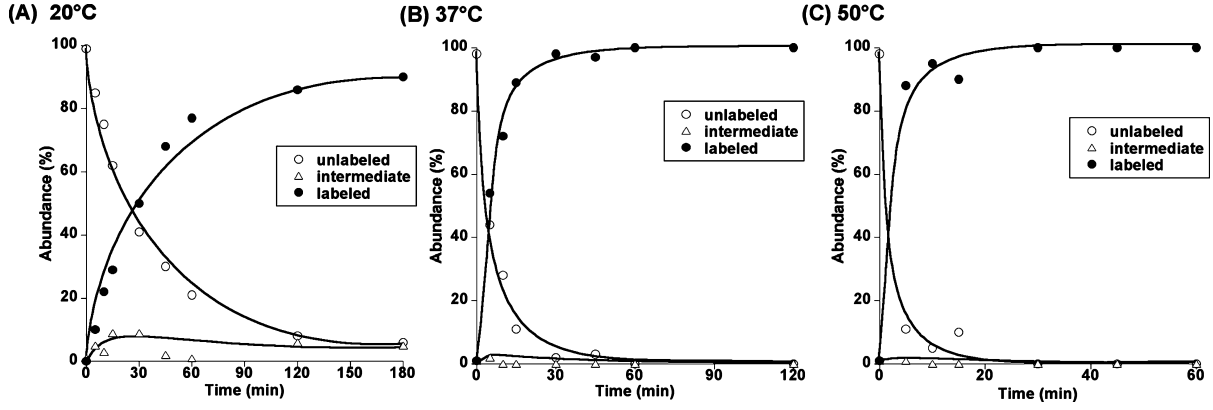

Figure 3. Time course of the derivatization reaction at reaction temperatures of 20 °C (A), 37 °C (B), and 50 °C (C).

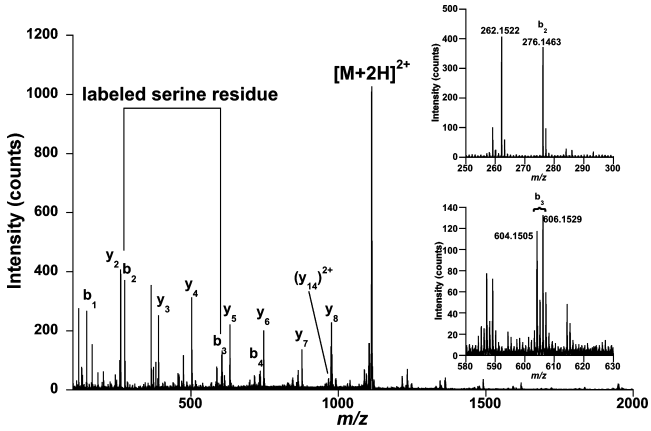

Figure 4. Product ion mass spectrum $[M + 2H]^{2+}$ of derivatized peptide fragment (amino acids 33−48) from β-casein. MS conditions: instrument, $QSTAR_{XL}$; flow rate, 200 nL/min; solvent, water/methanol/formic acid (50:50:1, v/v/v); ion spray voltage, 3000 V; declustering potential, 60 V; collision energy, 50 eV; collision gas (N_2), 3 units.

temperature for 30 min. After evaporation of the organic solvent, diethyl ether (600 mL) and 1% aqueous sodium hydroxide (200 mL) were added to the residue to extract the disulfide dimer (**3**), and this step was repeated four times. The residue obtained from evaporation of the combined organic layers was recrystallyzed from ethyl acetate to afford the disulfide dimer as white crystals (**3**, 727 mg).

A solution of tris(2-carboxyethyl)phosphine (345 mg, 1.2 mmol) in water (3 mL) was added to a solution of the disulfide dimer (**3**, 150 mg, 0.3 mmol) in tetrahydrofuran (12 mL). The reaction mixture was stirred at room temperature overnight and then concentrated to remove tetrahydrofuran. The aqueous layer was extracted with ethyl acetate and the extract was concentrated to afford *N*-(4-bromobenzoyl)aminoethanethiol as white crystals (**4**, 159.2 mg, 0.6 mmol). The total yield was approximately 56%, and any impurity spots were not observed on TLC (*n*-hexane/ethyl acetate, 1:1). HR-MS (FAB): *m/z* calculated for $C_9H_{11}ONSBr$ $[M + H]^+$ 259.9745, found 259.9729. ^1H NMR (500 MHz, CD_3COCD_3): δ 8.09 (br s, 1H), 7.83 (d, $J = 8.5$ Hz, 2H), 7.63 (d, $J = 8.0$ Hz, 2H), 3.54 (q, $J = 6.3$ Hz, 2H), 2.74 (q, $J = 7.5$ Hz, 2H). 7.83 (t, $J = 8.4$ Hz, 1H).

Derivatization of Protein. The protein (5 nmol) was dissolved in 30 μL of 1.65 mol/L Tris-HCl buffer (pH 8.6) containing EDTA (0.03 mol/L) and guanidine hydrochloride (7.3 mol/L). After the addition of dithiothreitol in the same buffer (38 μg, 1 mg/mL), the mixture was incubated at 50 °C for 3 h under a nitrogen atmosphere. Iodoacetamide in the same buffer (23 μL, 10 mg/mL) was added and the reaction mixture was incubated at room temperature for 30 min under a nitrogen atmosphere in the dark. The reduced and alkylated protein was lyophilized after dialysis using a mini dialysis unit (PIERCE, Rockford, IL).

To a solution of the reduced and alkylated protein (100 pmol) in water (20 μL) were added a solution of *N*-(4-bromobenzoyl)aminoethanethiol (100 nmol) in dimethyl sulfoxide (10 μL) and saturated aqueous barium hydroxide (20 μL), and the mixture was incubated at 37 °C for 2 h. The reaction was terminated by adding 10% aqueous TFA (10 μL), and the mixture was diluted with 200 μL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 1 mmol/L EDTA. The solution was applied onto a Thiopropyl Sepharose 6B column (33 mg, Amercham Biosciences, Uppsala, Sweden), and then 250 μL of the same buffer was added onto the column. The eluant was collected and sequentially dialyzed against 50 mmol/L ammonium bicarbonate/ethanol (9:1, v/v).

A solution of trypsin in 50 mmol/L aqueous ammonium bicarbonate (2 pmol/10 μL) was added to the protein solution and the mixture was incubated at 37 °C for 24 h. The reaction

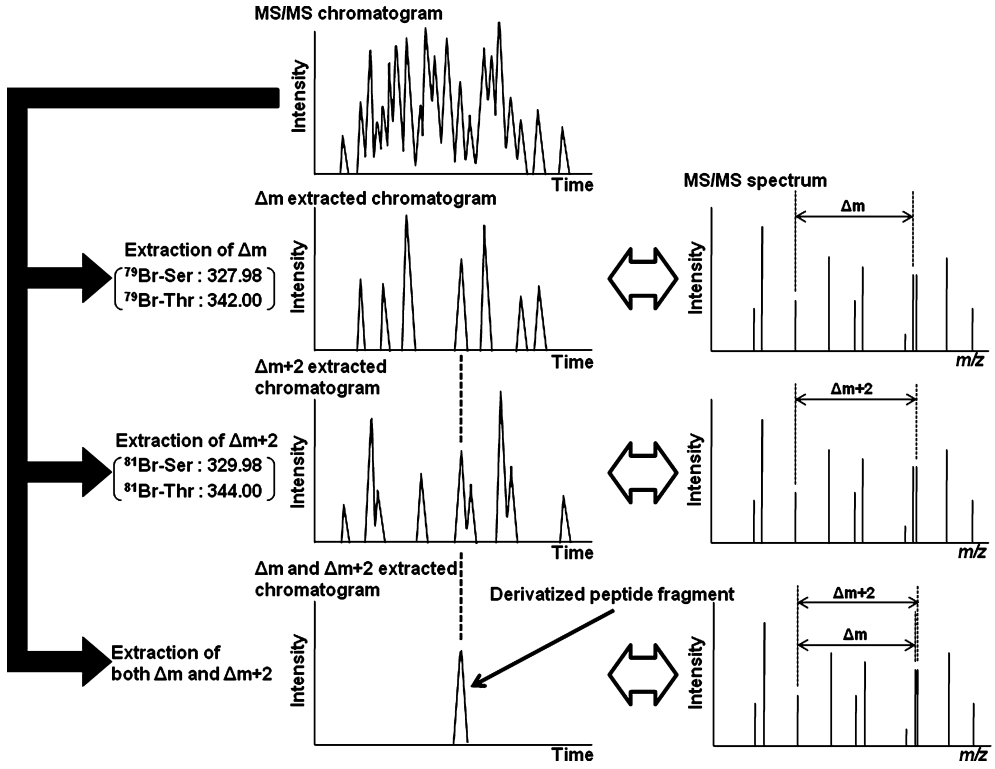

Figure 5. Strategy for double pseudoneutral loss extraction.

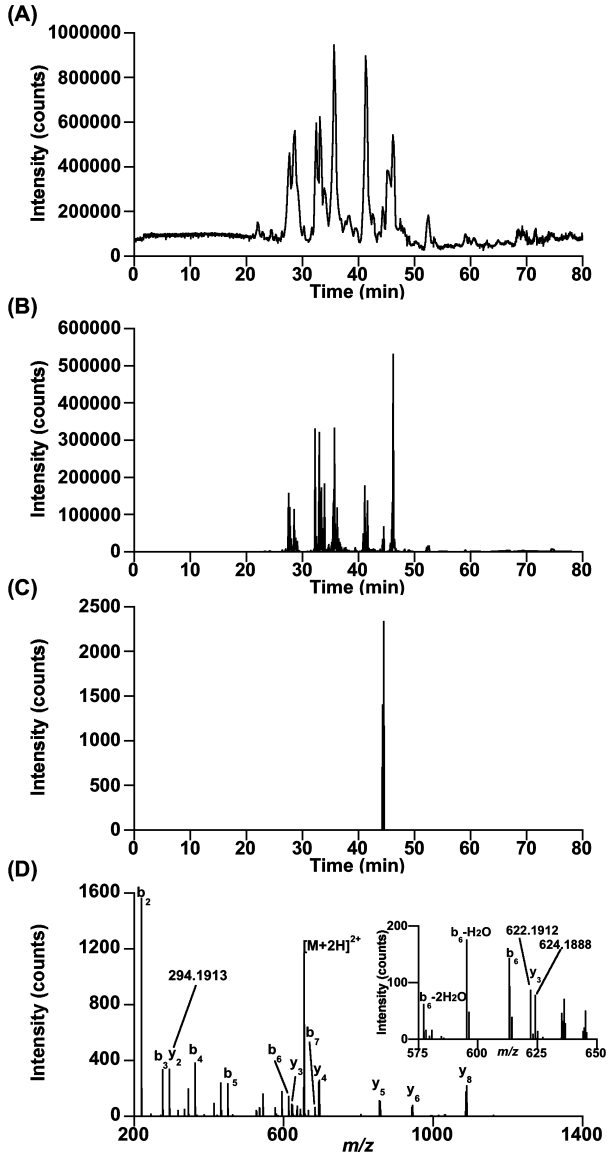

Figure 6. Analytical results for a mixture of a derivatized synthetic phosphorylated peptide (FAGSSYApSFK) and excess trypsin-digested BSA. (A) Total ion chromatogram for MS. (B) Total ion chromatogram for MS/MS. (C) Double neutral loss extraction chromatogram for 327.98 and 329.98 mass units. (D) Product ion mass spectrum of $[M + 2H]^{2+}$ of derivatized synthetic phosphorylated peptide.

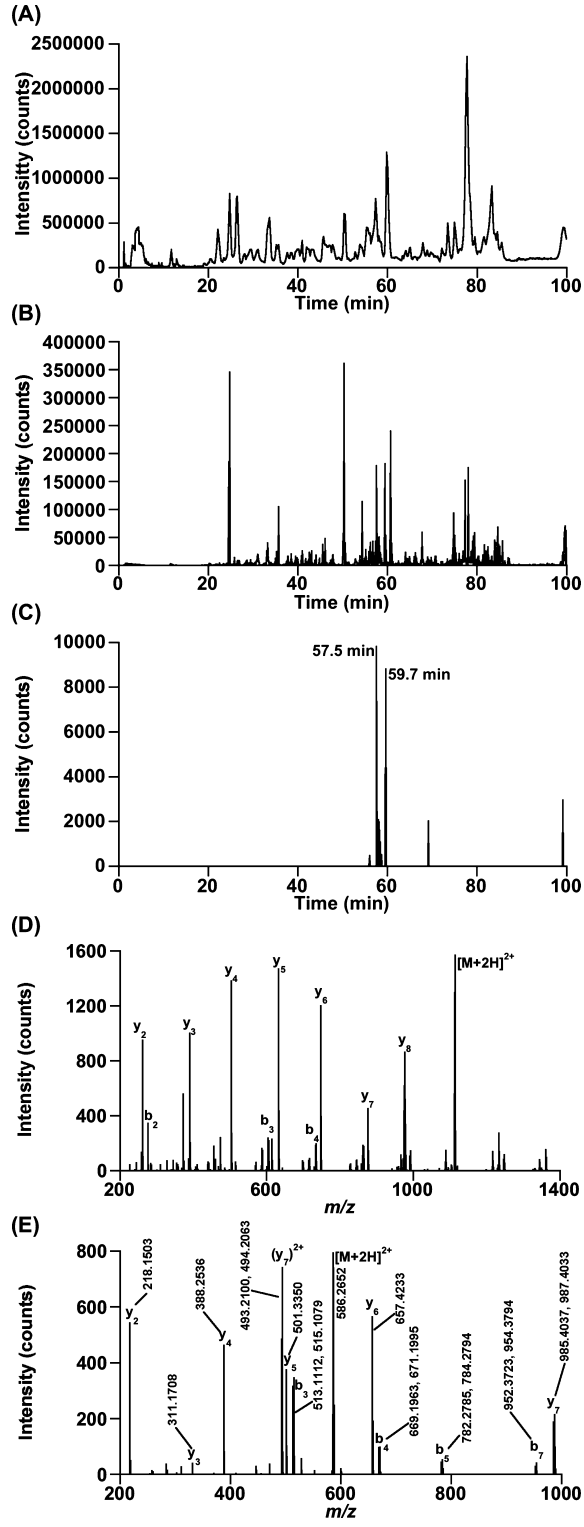

Figure 7. Analytical results for the tryptic digest of a mixture of 10 different derivatized proteins including β-casein. (A) Total ion chromatogram for MS. (B) Total ion chromatogram for MS/MS. (C) Double neutral loss extraction chromatogram for 327.98 and 329.98 mass units. Product ion mass spectrum of $[M + 2H]^{2+}$ of an extracted peak at the retention time of (D) 57.5 min and (E) 59.7 min.

was terminated by adding 10% aqueous TFA (100 μL), and then the sample was dissolved in water/methanol/formic acid (98:2:0.1, v/v/v) and injected into the nanoLC/ESI-MS/MS.

RESULTS AND DISCUSSION

The derivatization agent *N*-(4-bromobenzoyl)aminoethanethiol was prepared according to the synthetic route shown in Figure 1A. Since the free mercapto group is highly reactive, we chose to generate the desired thiol by reductive cleavage of the disulfide bond in the relatively stable homodimer. The homodimer (**3**) was produced by slow addition of cystamine hydrochloride to an excess of *p*-bromobenzoic acid activated ester (**2**) in the presence of triethylamine. The structure of the homodimer was confirmed by the fast atom bombardment mass spectrum, which showed a characteristic isotopic pattern of three peaks with an intensity ratio of approximately 1:2:1 and a peak-to-peak separation of two mass units (data not shown). After reductive cleavage of the disulfide bond in the homodimer (**3**), the new derivatization reagent (**4**) was obtained. The fast atom bombardment mass spectrum of the product included the characteristic signals for bromine, with the

^{79}Br-containing monoisotopic peak observed at m/z 260 and the ^{81}Br-containing monoisotopic peak of similar intensity at m/z 262 (data not shown). ^{1}H NMR data also confirmed the structure of this derivatization reagent.

Derivatization of peptides with *N*-(4-bromobenzoyl)aminoethanethiol proceeds in two steps, as shown in Figure 1B. A phosphorylated peptide derived from β-casein (amino acids 33−48, FQpSEEQQQTEDELQDK) was selected as a model peptide. The fragment was isolated by HPLC fractionation after tryptic digestion of β-casein. As a test reaction, a mixture of the model peptide (1 nmol) in water (20 μL) and saturated aqueous barium hydroxide (20 μL) was incubated at 50 °C for 2 h with or without the addition of a solution of *N*-(4-bromobenzoyl)aminoethanethiol (9.75 μmol) in ethanol (7.5 μL). The reaction mixture was injected into an HPLC system, and the product peaks were collected and analyzed by MS.

MALDI-TOF mass spectra of the starting material (**5**), the β-eliminated product (**6**), and the final product (**7**) are provided in Figure 2. The β-eliminated product peak was clearly observed at m/z 1963.88, and the 98 mass unit decrease from the starting material (**5**) corresponded to a loss of H_3PO_4 (Figure 2A,B). The mass difference between the β-eliminated intermediate (**6**) and the final product (**7**) was consistent with the expected increase resulting from the Michael addition of *N*-(4-bromobenzoyl)aminoethanethiol to the β-eliminated intermediate (**6**) (Figure 2B,C). The results from the test reactions with the model peptide provided evidence that the derivatization of a phosphorylated peptide using *N*-(4-bromobenzoyl)aminoethanethiol produced the desired compound.

The derivatization procedure can be run in one pot because the second step of the sequence proceeds very quickly. The phosphorylated peptide (amino acids 33−48 of β-casein) used as a model compound in the proof of concept study was also used to optimize reaction conditions for both steps of the derivatization procedure. The first step of the sequence, β-elimination of phosphoryl groups, typically occurs under strongly basic conditions. However, the use of a highly concentrated sodium hydroxide solution has been shown to result in β-elimination of not only the phosphoryl group but also of *O*-linked sugar chains.[21] Byford et al. reported that the use of barium hydroxide solution results in rapid and specific β-elimination of phosphoryl groups, without the nonspecific eliminations of other groups observed with sodium hydroxide solution.[22] Therefore, saturated barium hydroxide solution was selected as the base for β-elimination of phosphoryl group in the first step of the derivatization sequence. The barium hydroxide solution was generally added into the peptide solution to give a final concentration of $Ba(OH)_2$ of approximately 0.15 mol/L. With the optimal base for efficient β-elimination selected, we turned to optimization of the reaction temperature.

The effect of reaction temperature on the derivatization procedure was investigated, and the results are shown in Figure 3. The reaction course was evaluated at 20, 37, and 50 °C. The starting material gradually decreased over time under every temperature condition, but higher temperatures resulted in a more rapid reaction progress. The reaction intermediate, the β-eliminated peptide (**6**), was barely detected in the 50 °C experiment (less than 1%), but it was detected at a level of approximately 10% in the 20 °C experiment. The amount of final product (**7**) increased proportionately with the decrease of the starting material at all tested temperatures, but full conversion to the final product (**7**) did not occur at 20 °C after 180 min. In contrast, nearly complete conversion of the phosphopeptide to the final product was observed after 60 min at 37 °C or after 20 min at 50 °C.

The product ion mass spectrum of the final product ($[M + 2H]^{2+}$) derived from derivatization of a phosphorylated peptide (amino acids 33−48 of β-casein) is shown in Figure 4. The y_2, y_3, y_4, y_5, y_6, y_7, and y_8 ions and b_2, b_3, and b_4 ions confirmed the structure of the derivatized peptide. The b_3 and b_4 ions, which include the labeled serine residue, had a characteristic isotopic pattern with an intensity ratio of approximately 1:1 and a two mass unit difference between peaks, as shown in the expansion of the b_3 peaks in Figure 4. However, the b_2 ion, which does not include the labeled serine residue, showed a natural isotopic pattern. The characteristic pattern, indicating the presence of bromine from the derivatization agent, is very useful for identifying phosphoserine and phosphothreonine residues in peptides.

In addition, the characteristic isotopic pattern can be used for the detection of derivatized peptides in a complex peptide mixture. The MS/MS chromatogram of an enzymatically digested protein mixture is usually complicated because of the presence of a wide variety of peptides. The common single pseudoneutral loss extraction on a hybrid tandem mass spectrometer with standard resolution mode was not enough for the selection of target peptides[23] because of the existence of many nonspecific peaks. Fortunately, in the case of the present derivatization approach, the product ions possessing derivatized serine or threonine residues show a characteristic isotopic pattern, and the residue-free product ions have a normal isotopic pattern as described above. Therefore, in the selection of the derivatized peptide, double pseudoneutral loss extractions can be performed. For the phosphorylated serine residue, the pseudoneutral loss extractions are ^{79}Br-Ser at 327.98 and ^{81}Br-Ser at 329.98, and for the phosphorylated threonine residue, the pseudoneutral loss extractions are ^{79}Br-Thr at 342.00 and ^{81}Br-Thr at 344.00. The extraction of the product scan mass spectrum by targeting double pseudoneutral losses can significantly increase selectivity (Figure 5).

To validate the utility of this strategy for the specific extraction of a derivatized peptide, a derivative produced from a synthetic peptide (FAGSSYApSFK) was mixed with a 10-fold molar excess of bovine serum albumin tryptic digest, and the mixture was analyzed by nanoLC/ESI-MS/MS. The MS/MS chromatogram (Figure 6B) included many intense peaks corresponding to peaks on the MS chromatogram (Figure 6A), and the derivatized synthetic peptide was difficult to detect. On the other hand, the extracted chromatogram (Figure 6C), which was generated by double pseudoneutral loss extraction, showed only two peaks at retention times of 44.3 and 44.5 min. Both peaks had very similar product ion mass spectra because nucleophilic attack to α,β-unsaturated ketone creates two diastereoisomers.[24] The peak

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intensity was less than two hundredth of the most intense peak on the MS/MS chromatogram, and the result suggested that the double pseudoneutral loss extraction strategy effectively selected minor peaks of interest from a complicated chromatogram. The product ion mass spectrum of the doubly charged protonated molecule is shown in Figure 6D. The y_3 ion consists of two peaks with nearly equal intensity and a two mass unit difference, and the mass difference between the y_2 and y_3 ions is consistent with a derivatized serine residue. Although cysteine is sometimes modified to dehydroalanine by heat and alkali treatments,[25] any conversion of cysteine residues in BSA molecule did not appear under the present reaction conditions.

To further demonstrate the practicality of our approach, we applied the derivatization method to the analysis of a phosphorylated protein in a complex protein mixture. Equimolar amounts of β-casein, bovine serum albumin, immunoglobulin G, α-chymotrypsinogen, catalase, carbonic anhydrase, transferrin, plasmin, myoglobin, and lysozyme were mixed and then reduced and alkylated sequentially using dithiothreitol and iodoacetamide in the presence of a high concentration of guanidine hydrochloride. After removal of all reagents by dialysis, the mixture was incubated with *N*-(4-bromobenzoyl)aminoethanethiol in the presence of ca. 0.15 mol/L barium hydroxide. To remove large excess amounts of the derivatization reagent, the reaction mixture was applied onto an activated thiol gel column, and the nonretained fraction was collected, digested with trypsin, and analyzed by nanoLC/ESI-MS/MS. Detection of the derivatized peptide was very difficult because of the presence of many intense peaks on both the MS (Figure 7A) and MS/MS (Figure 7B) chromatograms. The auto MS/MS analysis contained 962 product ion mass spectra. As shown in Figure 7C, application of the double pseudoneutral loss method for derivatized serine residues extracted only a few weak peaks with less than a 40th of the intensity of the most intense peaks in the MS/MS chromatogram. Among them, two peaks at retention times of 57.5 and 59.7 min were detected as relatively intense peaks. The peak at 57.5 min had almost the same product ion mass spectrum as that of the derivatized form of amino acids 33−48 from β-casein, including the expected characteristic isotopic pattern for bromine (Figure 7D). In addition, the later peak (59.7 min) also contained the characteristic isotopic pattern of two peaks with about 1:1 intensity ratio and two mass unit difference, and the product ion mass spectrum suggested that the peptide possesses pSRL(I)GL(I) as a partial sequence (Figure 7E). This partial sequence is involved in hypothetical protein AN4196.2 in *Aspergillus nidulans* FGSC A4 by NCBI database, and the result of the product ion mass spectrum agrees completely with amino acids 101−109, AISRLGLAK, suggesting that Ser^{103} is phosphorylated, although the reason for commingling of this protein with the protein mixture is unclear. On the other hand, the double pseudoneutral loss extraction for derivatized threonine residues gave us a simpler chromatogram, which included only one peak of the retention time at 57.5 min (data not shown). However, the retention time of the peak was the same as the derivative of amino acids 22−48 from β-casein, and the mass difference between b_3 (*m/z* 604 and 606) and y_2 (*m/z* 262) agreed accidentally with the theoretical double pseudoneutral loss (342.00 and 344.00) of a derivatized threonine residue. The results from this study suggest that the present strategy may have applicability for the analysis of phosphorylated serine and threonine in complex mixtures of phosphorylated proteins.

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CONCLUSIONS

In this paper, we described the development of a new derivatization reagent, *N*-(4-bromobenzoyl)aminoethanethiol, useful for the selective analysis of phosphorylated proteins. This reagent reacts with α,β-unsaturated ketones produced by the β-elimination of phosphoryl groups from phosphorylated serine and threonine residues. The product ion mass spectra of the derivatized thioether products possess a characteristic isotopic pattern in which the derivatized residue-containing fragment ions have twin peaks with an intensity ratio of approximately 1:1 and a difference of two mass units. These characteristics permit the effective identification of phosphorylated serine and threonine residues in peptides. Moreover, derivatized peptides can be detected in a complicated peptide mixture by use of the double pseudoneutral loss extraction method and analysis of the extracted peaks for the presence of the characteristic isotopic pattern. The novel analytical method will be useful for the identification of phosphorylated serine and threonine residues in proteins.

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