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N-Terminal Derivatization of Peptides with Isothiocyanate Analogues Promoting Edman-Type Cleavage and Enhancing Sensitivity in Electrospray Ionization Tandem Mass Spectrometry Analysis

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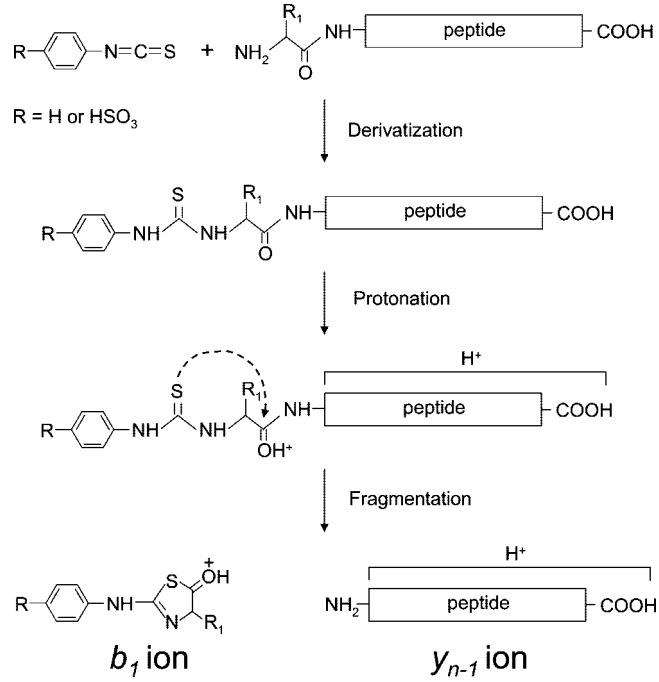
N-terminal derivatization of peptides with Edman's reagent, phenyl isothiocyanate (PITC), promotes gas-phase Edman cleavage that yields abundant complementary b_1 and y_{n-1} ion pairs by tandem mass spectrometry (MS/MS). The formation of b_1 ions can be utilized as a mass tag to enhance the interpretation of MS/MS spectra and increase the confidence of peptide identification during mass spectrometry analysis. Derivatization of tryptic peptides with another isothiocyanate analogue, 4-sulfophenyl isothiocyanate, also produces signature ions resulting from Edman cleavage and facilitates peptide sequencing on linear or branched peptides. The limitation of these derivatizations, however, is reduced MS signal intensities of modified peptides, due presumably to the tags themselves. Here we have demonstrated that several other isothiocyanate analogues bearing basic moieties can derivatize peptides and significantly improve the MS sensitivity of tagged analytes, while promoting Edman fragmentation and maintaining other sequence fragments as well.

Chemical derivatization of protein digests has been used to facilitate peptide sequencing via tandem mass spectrometry (MS/MS) in the "bottom-up" proteomic approach. MS/MS analysis of derivatized peptides can elevate ionization efficiency, induce favorable fragmentation patterns, or generate specific signature fragments. As consequence, increased sensitivity of detection and improved de novo sequencing of peptides can lead to highly confident identification of proteins.[1−4]

Edman degradation is a widely used method for the N-terminal sequencing of protein and peptides.[5] The N-terminal amino acids of analytes can be determined by sequentially analyzing modified N-terminal residues cleaved from a thiocarbamoyl derivative labeled with Edman's reagent, phenyl isothiocyanate (PITC). To understand the mechanism of peptide fragmentation induced by collision energy during MS/MS analysis, the decomposition of PITC-derivatized peptides was investigated by Summerfield et al.[6] It has been observed that collision-induced dissociation of a doubly charged peptide derivative resulted in a pair of abundant complementary b_1 and y_{n-1} ions (Scheme 1). This and additional experimental evidence lead to the conclusion that gas-phase fragmentation of a peptide modified by Edman's reagent proceeds by a mechanism similar to that of condensed-phase Edman degradation, where the preferential cleavage of the first peptide

Scheme 1. Preferential Gas-Phase Edman Cleavage of the Peptide Derivatized by Phenyl Isothiocyanate (PITC) or 4-Sulfophenyl Isothiocyanate (SPITC)



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bond is promoted by nucleophilic attack of the thiocarbonyl moiety bearing on the tag on the carbonyl group of the N-terminal peptide bond.

Gas-phase Edman cleavage and the formation of b_1 ions, typically not observed in the fragmentation of protonated peptides, can be utilized to facilitate confident protein identification for proteomics.[7,8] The ready identification of N-terminal residue adds an additional constraint to database searching that could further enhance interpretation of tandem mass spectra and increase the confidence level of MS-based peptide sequencing. It has been demonstrated that PITC derivatization approach can achieve significant gain in database searching efficiency for peptide identifications in yeast proteome.[9] In a direct analysis of a mixture of PITC-derivatized tryptic peptides by Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, van der Rest et al. demonstrated that as few as one peptide derivative could give rise to correct identification based on peptide mass and N-terminal residue identity.[7] Beardsley et al. investigated the possibility of utilizing b_1 ions generated from amidinated peptides as internal calibrants for accurate mass measurements.[10] Taking the advantage of the specific fragmentation of PITC derivatives, a multiple reaction monitoring (MRM) analysis using either b_1 or y_{n-1} as selected product ion has been attempted in the detection of prion protein.[11]

PITC is not the only chemical that induces gas-phase Edman degradation. It was reported by several groups that a labeling reagent, 4-sulfophenyl isothiocyanate (SPITC), used for N-terminal sulfonation, also led to the dominant formation of y_{n-1} ions resulting from the fragmentation of derivatized peptides.[12,13] Since the reagent has a structure similar to PITC, and it attaches to peptides by the same chemistry as the latter does, it was believed that SPITC-derivatized peptides also undergo preferential Edman-type cleavages even though it is almost impossible to detect b_1 ions because the positive charge of this ion is neutralized by negative charge of the sulfonic acid group (Scheme 1). Gas-phase Edman degradation has been found to be particularly useful in characterizing branched peptides. Utilizing SPITC derivatization, Wang and co-workers have recently reported a novel strategy for identifying protein ubiquitination sites with high efficiency and specificity using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry and electrospray ionization (ESI) mass spectrometry.[14−16] In this method, two tags are added to the diglycine-branched peptides resulting from trypsin digestion of ubiquitin-modified proteins and thus lead to the formation of a unique group of signature fragments that can be used to distinguish these branched peptides from others.

Despite their success in peptide sequencing and protein identification, some inherent properties of PITC and SPITC limit their application. The major problem is reduced sensitivity of MS detection, presumably due to aromatic or sulfonic acid groups of these reagents. The negative charge feature of sulfonic acid group is the apparent cause of a 10-fold decrease in positive mode mass detection of SPITC-derivatized peptides.[13] Further decrease in sensitivity could be expected when two sulfonation tags are introduced into a peptide as in the case of branched peptide. The decrease in ionization of PITC-modified peptides is also expected because the basic amino group is blocked by nonpolar phenyl group. The loss of PITC-derivatized peptide was reported due to the precipitation of some modified peptides.[7] In addition, it was observed in our laboratory that excess SPITC reagent could be eluted out together with some peptides during the separation by reversed-phase chromatography, presumably due to the hydrophobicity of phenyl group, and thus could suppress the detection of these peptides.

Here we report a study of chemical derivatization with four novel isothiocyanate analogues in an attempt to overcome problems with PITC and SPITC described above. All of these compounds turned out to be effective in the derivatization of model peptides and in promoting gas-phase Edman cleavage. Most significantly, modified peptides derivatized with these reagents displayed higher sensitivity in mass detection than those derivatized by PITC and SPITC.

EXPERIMENTAL SECTION

Chemicals. All chemicals used in this study were of analytical grade. Phenyl isothiocyanate, 4-sulfophenyl isothiocyanate, 3-pyridyl isothiocyanate (PYITC), 2-piperidinoethyl isothiocyanate (PEITC), 3-(4-morpholino) propyl isothiocyanate (MPITC), 3-(diethylamino) propyl isothiocyanate (DEPITC), and bovine serum albumin (BSA) were obtained from Sigma Company (St. Louis, MO).

Peptide Synthesis. Peptides were prepared with Fmoc chemistry using solid-phase peptide synthesis method on an AAPPTEC APEX 396 multiple peptide synthesizer (Louisville, KY). Synthetic peptides were purified by reversed-phase (C-18) high-performance liquid chromatography using a gradient of water−acetontitrile solvent system containing 0.1% trifluoroacetic acid (TFA). The correct structures of the peptides were confirmed by mass spectrometry.

Chemical Derivatization. The peptide (100 pmol) was reacted with derivatization reagent (10 nmol) in 10 μL of solution containing pyridine, ethanol, and ddH_2O (1:1:1 vol/vol/vol) at 55 °C for 60 min. The reaction was terminated by adding 1 μL of 1% TFA. After reaction, 1 μL of the solution was transferred to a new 0.6 mL Eppendorf tube and dried completely by SpeedVac for mass spectrometry analysis. For BSA, 200 μg of the protein was digested with trypsin (50:1 w/w) in 30 μL of reaction solution containing 25 mM ammonium bicarbonate at 37 °C for 18 h. An amount of 1 μL of the reaction (100 pmol/μL) was then mixed with 10 μL of PYITC (2 nmol/μL) solution

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and underwent derivatization following the same procedure described.

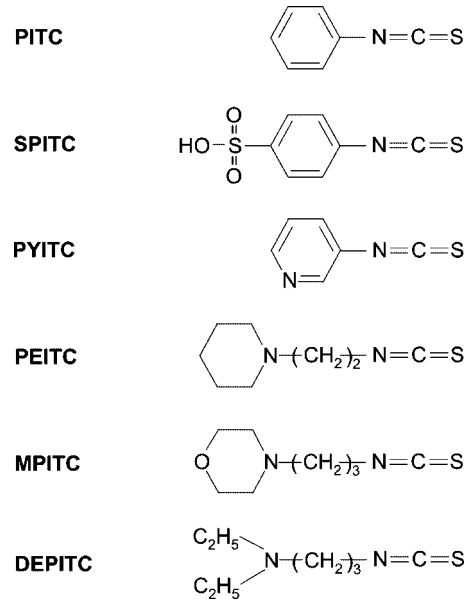
Mass Spectrometry. Derivatized peptide or peptide mixtures were resuspended in 50% methanol and 1% acetic acid to a final concentration of 0.5−1 pmol/μL and were introduced into the mass spectrometer by direct infusion at a flow rate of 0.4 μL/min. A potential of 3.00 kV was applied to the nanospray emitter in the ion source. The MS and MS/MS analysis was performed on a Micromass Q-Tof Ultima mass spectrometer (Waters, Milford, MA). All mass spectra were obtained in the positive-ion mode with different collision energies (10, 20, 30, 40, and 50 eV). In quantitative comparison experiments, the spectra were obtained by combining MS scans acquired within 1 or 2 min. The acquisition of data was performed on a MassLynx data system (version 4.0). Resulting MS/MS data were interpreted manually.

RESULTS AND DISCUSSION

In the selection of novel N-terminal derivatization reagents alternative to PITC and SPITC, several factors should be considered: promotion or induction of Edman cleavage in MS/MS fragmentation, enhancement of ionization efficiency of modified peptides, high modification efficiency, and low side reactions. For PITC- or SPITC-labeled peptides, the key element for gas-phase Edman-type cleavage seems to be the thiocarbonyl group formed from the reaction between the isothiocyanate group of the reagents and the N-terminal amino group of a peptide. In addition, the low ionization efficiency of such derivatives likely results not from the isothiocyanate but from the aromatic and sulfonic acid groups. For those reasons, we targeted on compounds of isothiocyanate. Therefore, we felt we should consider isothiocyanate analogues containing a basic moiety so that the overall basicity of peptide derivatives labeled with such reagents would be maintained and the ionization efficiency could remain as high or higher than that of its underivatized counterpart. On the basis of these criteria, we selected four commercially available isothiocyanate analogues, PYITC, PEITC, MPITC, and DEPITC, and examined the feasibility of using them as derivatization reagents (Scheme 2). In addition to isothiocyanate structure, all of these reagents contain a tertiary amine group that is expected to maintain or elevate proton affinity of modified peptides for the enhancement of their mass sensitivity.

N-Terminal Derivatization of Model Peptides. A synthetic peptide, IYIGPGRAF, was reacted with six reagents, respectively, including four newly selected isothiocyanates and two old ones, PITC and SPITC, for comparison, under identical experimental conditions. As shown in Figure 1 and Table 1, new mass peaks corresponding to derivatized peptides were detected from all reaction mixtures by ESI mass spectrometry, indicating that selected reagents are all capable of modifying target analyte. The correct modification at the N-terminus of the peptide was confirmed by the masses of labeled peptides and tandem mass sequencing analysis (discussed below). The yields of derivatized peptides from the original ones ranged from 52−100%, based on the changes in the intensities of unmodified peptides in each individual case. Similar to unmodified peptide, doubly protonated species of the derivatives were dominant in the reaction products while singly charged forms were detected in some cases. These results demonstrate that the selected isothiocyanate analogues

Scheme 2. Structures of Isothiocyanate Compounds: Phenyl Isothiocyanate (PITC), 4-Sulfophenyl Isothiocyanate (SPITC), 3-Pyridyl Isothiocyanate (PYITC), 2-Piperidinoethyl Isothiocyanate (PEITC), 3-(4-Morpholino) Propyl Isothiocyanate (MPITC), and 3-(Diethylamino)propyl Isothiocyanate (DEPITC)



are capable of modifying peptides at their N-terminus in the same way as PITC and SPITC do.

It is not surprising to see that the peak intensities of PITC- and SPITC-tagged peptides were much smaller than that of the original peptide not undergoing derivatization, as determined in the control experiment (Table 1). Although the apparent conversion rate of 83% and 96% from PITC and SPITC reactions, respectively, seems to imply that the majority of the peptide was converted to modified derivatives, however, the combined intensities of doubly and singly charged ions of two derivatives are only 7% and 20%, respectively, of the intensity of the peptide before derivatization. There was no significant improvement when samples were desalted prior to MS analysis (data not shown). This is consistent with the previous observation that the derivatization by Edman's reagent PITC, or sulfonation reagent SPITC, dramatically reduces sensitivity in mass detection. In a previous study,[8] we had observed that sensitivity to SPITC derivatives could be influenced significantly by optimization of reaction conditions, but in every case intensities were less than that of the corresponding, underivatized peptide.

In contrast, derivatization with four newly selected isothiocyanate reagents, PYITC, DEPITC, PEITC, and MPITC, resulted in intense peaks of the modified peptides. In comparison with the data for PITC or SPITC reactions, more than 10-fold increases in the peak intensities of the derivatives were achieved in most cases, even though less peptide was modified under present conditions. These results suggest that the basic groups carried by these isothiocyanate analogues indeed enhance the ionization efficiency of the analytes of interest. Further improvement could be expected with the optimization on the derivatization procedure for individual reactions.

It has been observed by several groups (personal communications) including ours that a peptide containing an N-terminal

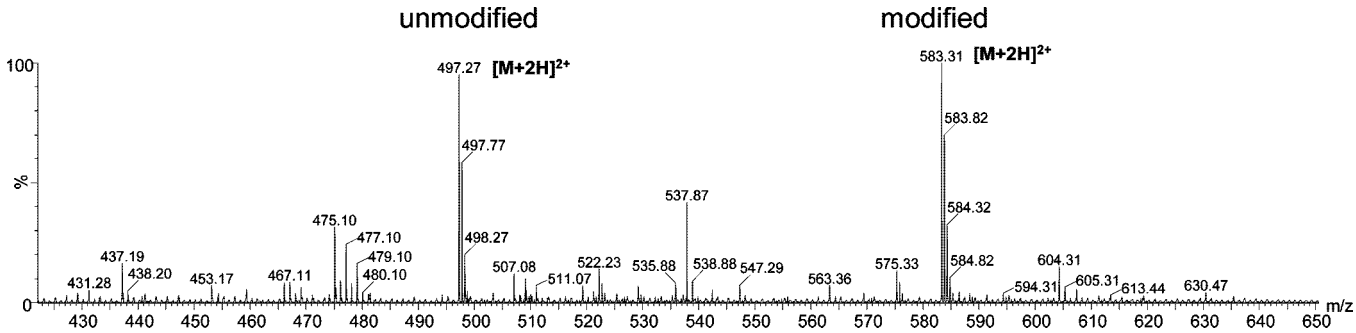


Figure 1. Mass spectrum of the peptide, IYIGPGRAF, after derivatization reaction with 3-(diethylamino)propyl isothiocyanate (DEPITC).

Table 1. Observed Peaks of a Synthetic Peptide, IYIGPGRAF, after Derivatization with Various Isothiocyanate Reagents[a]

	unmodified peptide				modified peptide		
derivatization reagents	mass (m/z)	charge	intensity (count)	conversion rate (%)[b]	mass (m/z)	charge	intensity (count)
SPITC	497.2	2+	809	96	615.7[c]	2+	1028
					1208.4	1+	945
PITC	497.2	2+	3176	83	575.7[c]	2+	497
					1128.5	1+	737
PYITC	497.2	2+	4628	75	565.2	2+	9374
					1129.5	1+	878
DEPITC	497.2	2+	8789	52	583.3	2+	9095
PEITC	497.2	2+	8052	56	582.3	2+	6616
MPITC	497.2	2+	10	100	590.3	2+	10711
control (underivatized)	497.2	2+	18367				

[a] At least three experiments were conducted for each derivatization reaction. [b] Conversion rate = $(\text{Int.}_{\text{control}} - \text{Int.}_{\text{reaction}})/\text{Int.}_{\text{control}}$. [c] Sodiated peptide with one sodium attached.

glutamic acid residue can form a truncated peptide (with the loss of its N-terminal amino acid residue) in addition to the desired derivative in the SPITC derivatization reaction. This could be caused by the participation of the carboxylic side chain of the glutamic acid in Edman-type cleavage, such that some of the derivatized peptides undergo preferential Edman degradation which allows an easy departure of SPITC-Glu from the molecule prior to collision-induced MS/MS environment. It is not clear whether the side reaction occurred in reaction solution or under MS condition during mass measurement. To test if this is a common phenomenon with N-terminal labeling by isothiocyanate analogues, we examined the reaction of a synthetic peptide, EGVNDNEEGFFSAR (GFP: Glu-fibrinopeptide, m/z 785.8, 2+), containing N-terminal glutamic acid residue, with our selected reagents. It turns out that all reactions yielded both a modified derivative and an unmodified N-Glu-truncated peptide at the mass of m/z 721.3 (2+), indicating that this side product is unavoidable (Figure 2). It was surprising to see that, in the reaction with PEITC, the major derivatization product was at m/z 806.3 (2+) which indicates that labeling occurred at the N-terminus of the truncated peptide instead of the intact one (Figure 2), i.e., that derivatization reoccurs on the truncated species after initial labeling and Edman degradation (there was a large excess of derivatization reagent.) Fortunately, the quantities of the modified peptides derivatized by the reagents containing basic groups are still dominant, and the formation of truncated peptide is relatively low (20% or lower). Given the low yield of the side product and the low occurrence of glutamic acid adjacent to lysine or arginine residues in proteins, the generation of N-Glu-truncated peptides caused by the side reaction during derivatization should not add significant interference with peptide sequencing and protein identification as a whole.

Tandem Mass Spectrometry of Peptide Derivatives. To explore the fragmentation behaviors of the peptide derivatives, a series of peptides, tag-IYIGPGRAF, modified by four selected isothiocyanate reagents were subjected to MS/MS analysis in the Q-Tof mass spectrometer with electrospray ionization at low collision energy (20 or 10 eV). Fragmentation of underivatized peptide yielded product ions from random peptide bond cleavage, but no b_1 ion was observed (Figure 3A). Although the y_{n-1} (y_8) ion was detected, its relative intensity is much lower than those of other products such as y_6 and y_7 ions. In contrast, the MS/MS of the four derivatized peptides all resulted in a pairs of y_8 (y_{n-1}) and b_1 ions, indicating that derivatizations with those reagents indeed promoted or induced preferential Edman-type cleavage during low-energy collision process in a way similar to PITC or SPITC derivatives. This cleavage occurs for the PYITC derivative under very low collision energy (10 eV), which does not usually induce meaningful fragmentation in an ordinary peptide of similar size and charge state. Interestingly, the fragmentation of PYITC derivative at 10 eV produced a pattern similar to those generated at 20 eV for other derivatives. Whether PYITC has high propensity compared to other reagents in inducing Edman cleavage needs further investigation.

Unlike PITC-derivatized peptides that usually fail to produce additional sequence fragments and are therefore limited for purposes of protein identification via MS/MS, the most interesting feature of our ITC derivatives is that they do not suppress the formation of other sequence fragments in MS/MS spectra, even while inducing N-terminal Edman cleavage. As shown in Figure

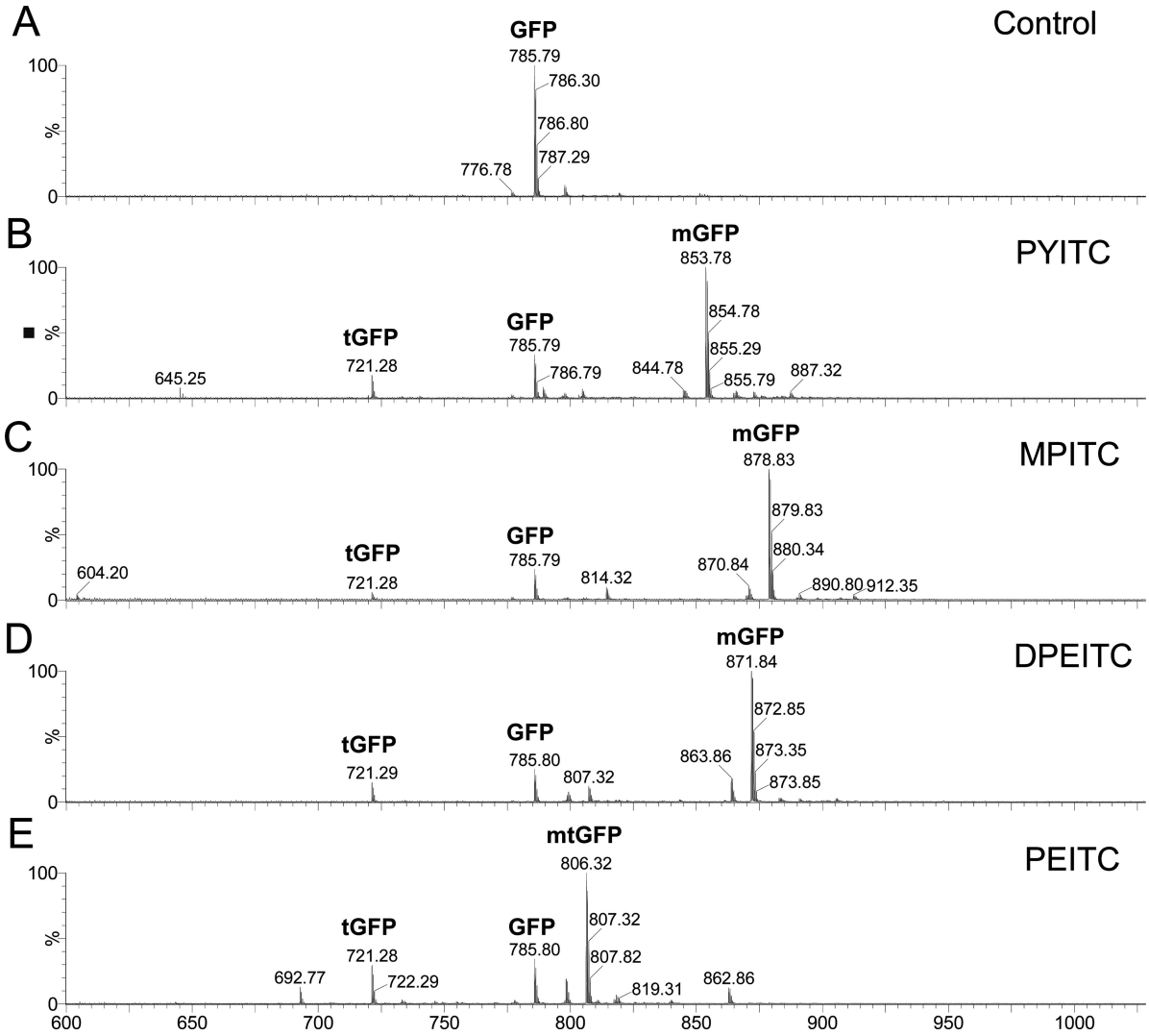


Figure 2. MS spectra of the peptide GFP, EGVNDNEEGFFSAR, before (A) and after derivatization with the reagent (B) PYITC, (C) MPITC, (D) DEPITC, and (E) PEITC. GFP and tGFP represent original and N-terminal Glu truncated peptides, respectively. mGFP and mtGFP stand for derivatives from intact and N-Glu-truncated peptides, respectively.

4, the MS/MS spectra of PYITC-derivatized peptide (GFP), at a collision energy of 30 and 40 eV, include all of the major product ions except b_2 seen with the underivatized peptide at a collision energy of 30 eV, plus the complementary b_1 and y_{13} ion pairs induced by preferential cleavage. In addition, the b_1 ion (m/z 266.0) of PYITC-derivatized GFP could be detected at various collision energies ranging from 10 to 50 eV, revealing the high stability of this characteristic ion. The formation of both Edman-type product and other sequence fragments during peptide fragmentation is a really significant advantage to peptide sequencing via MS/MS, because it allows the addition of another database searching constraint specifying the identity of the N-terminal amino acid residue, in a way analogous to that used with PITC-derivatized peptides,[8,9] while not sacrificing the information deduced from the fragmentation of other peptide bonds. Therefore, we can now truly say that the gas-phase Edman cleavage caused by peptide derivatization will facilitate protein identification.

In addition to Edman-type cleavage, the elimination of the entire tag group form the peptide derivatives was observed as another preferential cleavage under low collision energy. The pairs of intense peaks in the spectra (Figure 3B−E, Figure 4C−E) of the peptide derivatives represented protonated tag molecules (m/z 137.01, 173.10, 171.08, and 187.08) and their counterparts, [MH − tag] ions. The high intensities of these tag peaks presumably attribute to their high basicity. The existence of these tag ions could be used to validate chemical labeling.

Derivatization of Trypsin-Digested Protein. The application of novel reagents to the chemical derivatization of peptide mixtures resulting from digested proteins was examined. The result of derivatization of trypsin-digested BSA by PYITC is depicted in Figure 5, with the detailed Supporting Information in Table S-1. Peptide derivatives were determined by mass increases and confirmed by peptide sequencing by MS/MS analysis (data not shown). The modifications occurring on most of the tryptic peptides demonstrate the efficiency of this derivatization. The intensities of the majority of the derivatized peptides are comparable to those of their underivatized counterparts. Some peptide derivatives even showed higher intensities than their unmodified counterparts. Given the fact that the reaction mixtures were infused directly into the mass spectrometer without preliminary purification by liquid chromatography and along with the excess

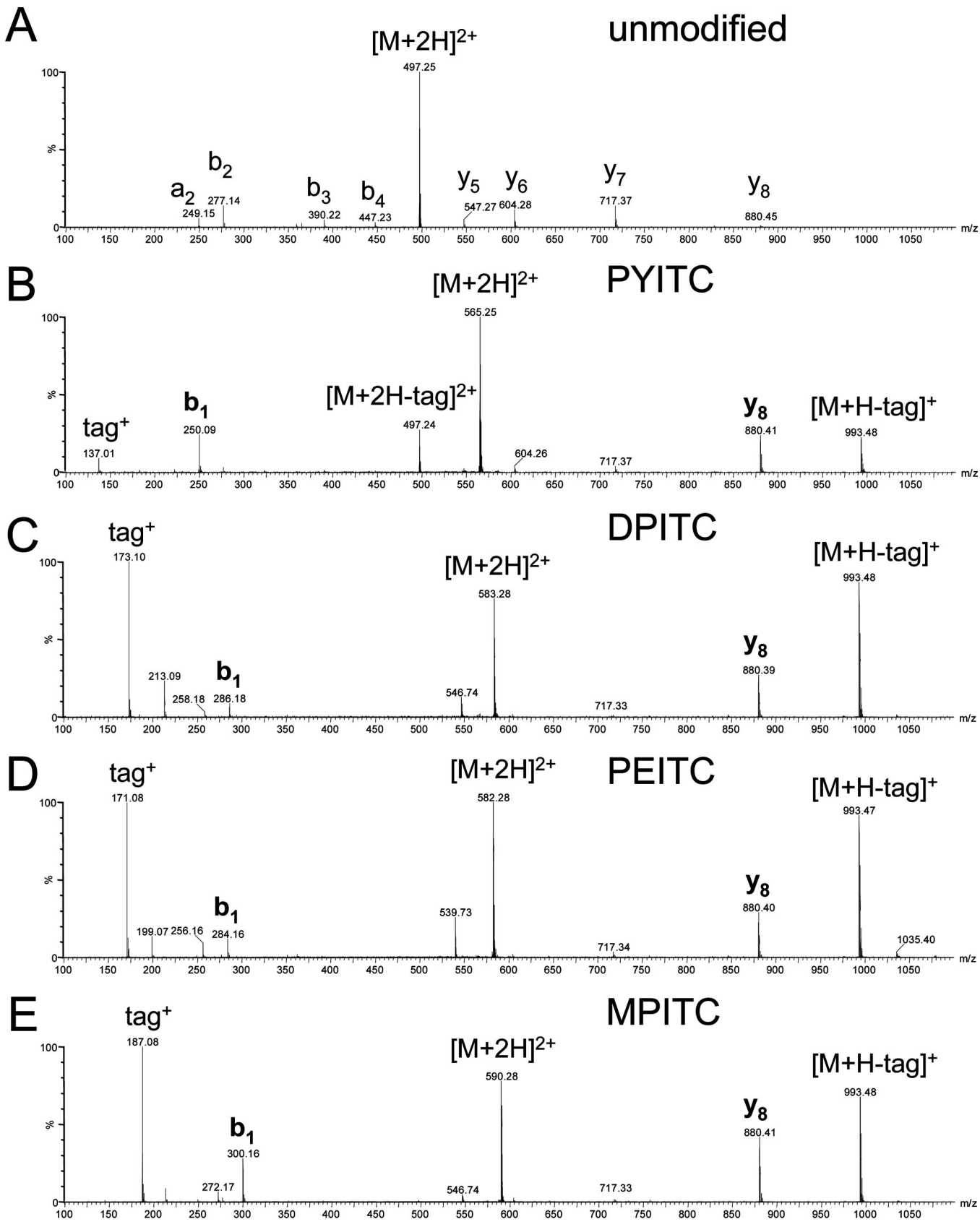


Figure 3. MS/MS spectra of the derivatives of the peptide, IYIGPGRAF: (A) underivatized, (B) PYITC, (C) DEPITC, (D) PEITC, and (E) MPITC. All but one of the experiments were carried out at 20 eV collision energy. The collision energy used for PYITC derivatives was 10 eV.

of unreacted reagent, one might suspect that the ionization efficiency of analyte to be adversely affected. These useful, higher intensities of peptide derivatives might be further optimized by deliberately adjusting the experiment conditions to that end.

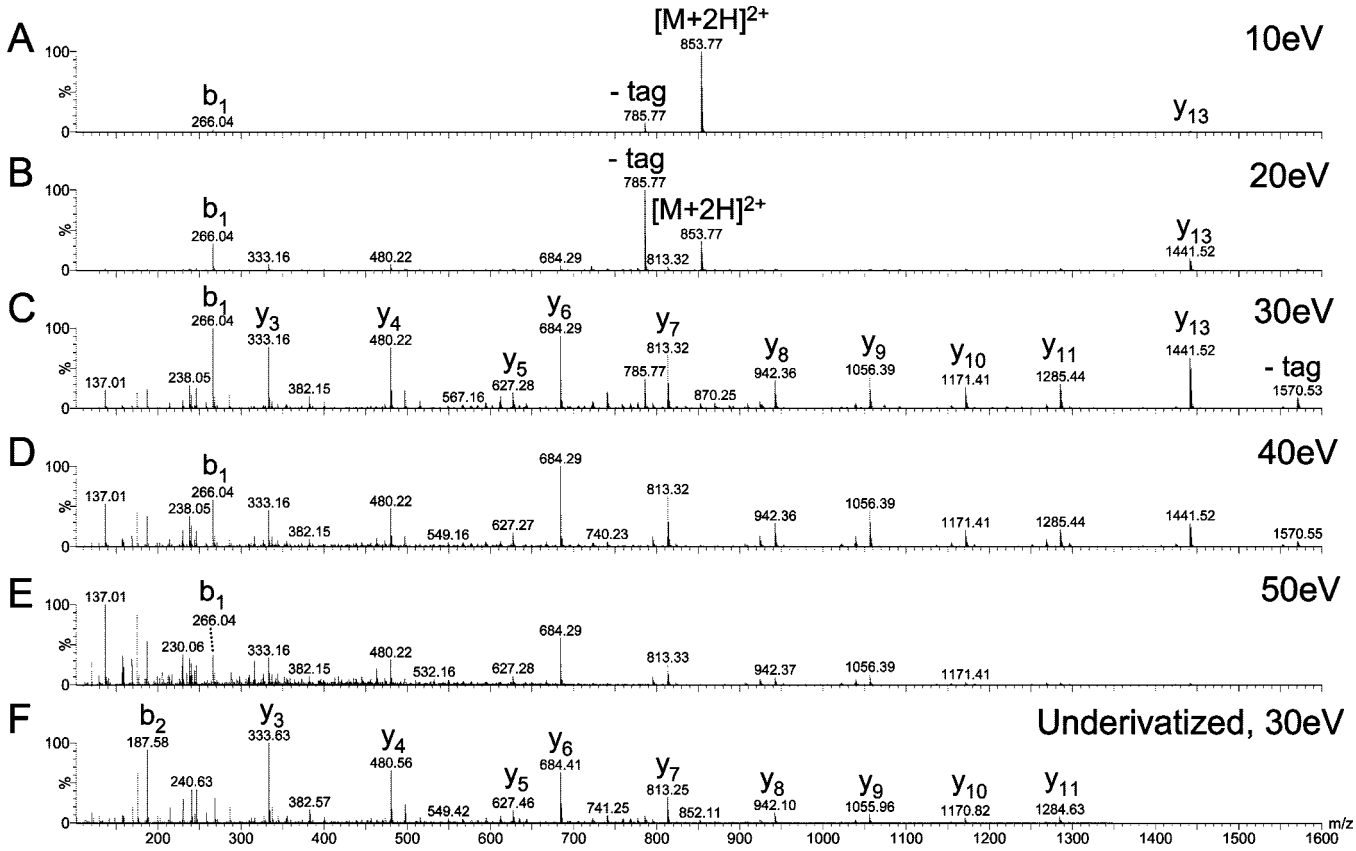


Figure 4. MS/MS spectra of PYITC-derivatized peptide (GFP) obtained at the collision energy of (A) 10, (B) 20, (C) 30, (D) 40, and (E) 50 eV and (F) the spectrum of underivatized peptide at 30 eV collision energy.

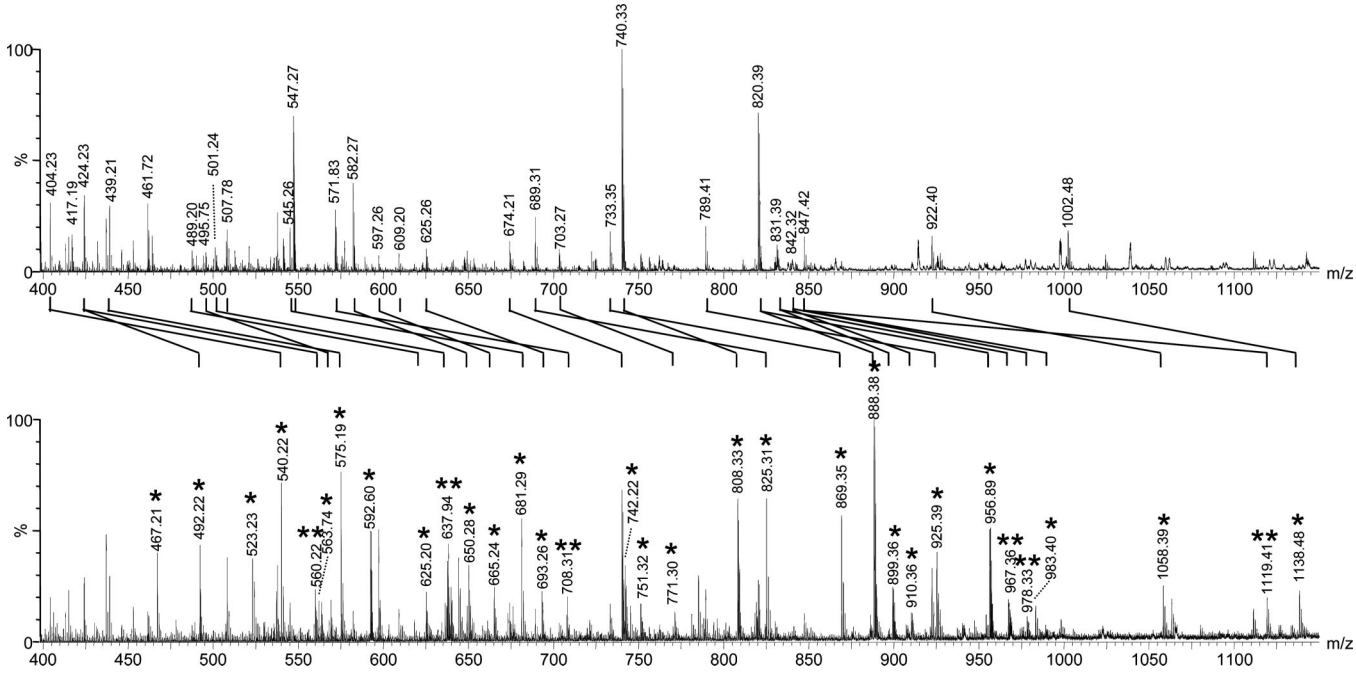


Figure 5. MS spectra of the peptide mixtures resulting from trypsin digestion of bovine serum albumin (top) before and (bottom) after chemical derivatization with PYITC. The peaks labeled with asterisks represent derivatized peptides. Double asterisks indicate the peptide was labeled by two PYITC tags, one on N-termini and another one on the amino group of a lysine side chain. The lines between two spectra connect underivatized peptides to their corresponding derivatives.

These results further confirm that the new derivatization method, versus conventional PITC and SPITC strategies, significantly increases sensitivity in mass detection. The existence of a few unmodified peptides and the occurrence of several doubly tagged peptides suggest the necessity for further optimization of the conditions of the derivatization reaction.

CONCLUSIONS

Reduced detection sensitivity of PITC- or SPITC-derivatized peptides by mass spectrometry likely come from the aromatic or sulfonic acid groups of these reagents. In order to improve MS signal intensity, we investigated four carefully selected isothiocyanate analogues that contain basic moieties for labeling peptides. It was observed that all of these reagents were able to label model peptides by the same mechanism as do PITC and SPITC. In comparison with PITC and SPITC derivatives, the MS signal intensities of the derivatives with these four new reagents increased at least 10-fold, implying that increased overall basicities of the derivatized peptides indeed enhance the ionization efficiency of the derivatized analytes. MS/MS analysis revealed that all of the peptides derivatized with each of the new reagents yielded complementary b_1 and y_{n-1} ion pairs, induced by preferential Edman-type cleavage during the low-energy collision process. While the pair of complementary of b_1 and y_{n-1} ions should lead to the identification of the N-terminal amino acid residue and to the addition of a database searching constraint, the other sequence product ions, which are comparable to these resulted from underivatized peptides, could be used to provide sequence information. The method was demonstrated with synthetic peptides and a mixture of peptides digested from a protein with trypsin. In conclusion, novel isothiocyanate derivatization reagents result in a significant improvement in mass detection of tagged analytes and should enhance the identification of proteins and peptides by promoting gas-phase Edman degradation. We have conducted promising preliminary experiments with a synthetic branched peptide derivative with a view to characterizing ubiquitinated proteins. Further studies are on the way to explore the potential application of the novel reagents on the direct identification and site mapping of proteins modified by ubiquitin, SUMO, and other ubiquitin-like molecules from simple or complex samples. The investigation on the application of this method in protein characterization and quantification is also ongoing in our laboratory.

SUPPORTING INFORMATION AVAILABLE

Supplementary table. This material is available free of charge via the Internet at http://pubs.acs.org.

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