

Short communication

Capillary electrophoresis tandem mass spectrometry of bromine-containing charged derivatives of peptides

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Abstract

Novel bromine-containing positively charged labels 5-bromo-1-ethyl-thiazolium (BET⁺) and 5-bromo-1-ethyl-pyridinium (BEP⁺) ions were studied for improving the interpretation of MS/MS spectra of peptides. 2,5-Dibromo-1-ethyl-thiazolium tetrafluoroborate (DBET) reacts in the order: ϵ - >> α -amino group >> hydroxyl group of Tyr while 2,5-dibromo-1-ethyl-pyridinium tetrafluoroborate (DBEP) reacts preferably with thiol group of Cys >> hydroxyl group of Tyr. In this study a simple and fast CE/MS/MS method is presented for investigating the labeling reaction with these new reagents, where the difference in migration times of labeled and unlabeled peptides also gives us information about the position of labeling. These bromine-containing reagents simplify the MS/MS spectra of peptides: the charge of the derivatives increases the intensity of the corresponding ions, thus enhancing the sensitivity of the detection and the characteristic distribution of the bromine isotope (the ⁷⁹Br and ⁸¹Br ratio is nearly one) facilitating the recognition. By eliminating the non-doubled peaks, clear and easily interpretable MS/MS spectra can be produced that contain only the labeled fragments.

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1. Introduction

The coupling of mass spectrometer (MS) with liquid-phase separation systems has great potential because it provides efficient separation and selective mass identification. Capillary electrophoresis (CE) is a fast, highly efficient separation method for small sample quantities; therefore, interfacing with electrospray (ESI) mass spectrometer became one of the most powerful techniques of separation and selective mass identification of charged analytes [1]. The capillary electrophoresis of peptides is possible using acidic volatile buffer which is compatible with electrospray mass spectrometry operated in positive ion mode. Under acidic condition, the electroosmotic flow in an uncoated fused-silica capillary is suppressed but the resulting low flow is compatible with the sheathless CE/MS interfaces. Owing to the properties described above, the CE/MS is a very efficient and unique tool in peptide analysis [2,16–18].

Peptide sequencing with tandem mass spectrometry (MS/MS) became [3] a widely used analytical method for characterization of proteins. The applicability of the MS/MS spectra for sequence determination depends highly on the nature of amino acids in the peptide. Recording MS/MS spectrum of the peptide that can be protonated only at the amide backbone (*e.g.* *N*-acetylated peptides) usually results in both *N*-terminal and *C*-terminal ions with similar abundance. In the presence of a basic site (free *N*-terminal amino group or basic amino acid, *e.g.* lysine or arginine) the charge is localized to the fragment where the basic site exists, increasing their abundances; hence the complexity of the spectrum is reduced. The chances of a complete sequence determination depend on the quality of spectra and also on a particular peptide sequence; thus developing a general method for peptide sequencing remains a challenge.

Peptide modifications for MS/MS were developed to simplify and to direct the fragmentation to form only *N*- or *C*-terminal ions in order to facilitate the interpretation of their mass spectra [4]. This goal can be achieved by different *C*-terminal [5] or *N*-terminal [6–9] modifications with charged or charge-retaining derivatives or derivatives that hide the charge of *N*-terminal amino group of tryptic peptides [10]. The charge is retained

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by the ion bearing the charged substituent; thus preponderantly, these ions are dominant in the MS/MS spectrum. If the spectrum contains *e.g.* only N-terminal ions, the mass differences between the same types of ions fit the masses of amino acid residues; hence a successful sequence determination is more likely. Introduction of a bromine atom in the charged derivatives further improves the chance of an easy and fast interpretation of the spectra by doubling the peaks due to the characteristic isotopic distribution of bromine [11].

2-Chloro-1-methylpyridinium iodide (Mukaiyama reagent) [12,13] and 2-bromo-3-ethylthiazolium tetrafluoroborate [13,14] were studied as coupling reagents in the synthesis of peptide nucleic acids (PNAs) in our laboratory. They gave low yields in oligomer synthesis as they were too reactive, and besides activating the monomer carboxyl group they reacted with the free N-terminal amino group leading to capped PNA chains. These side products were detected with high intensity in their mass spectra and that gave us the idea to exploit these coupling reagents for the introduction of positive charge for enhancing the sensitivity of the MS analysis. This favourable property was further enhanced by the introduction of a bromine atom into the labeling reagents leading to compounds 2,5-dibromo-1-ethyl-thiazolium and 2,5-dibromo-1-ethyl-pyridinium salts [14,15].

In this work, a simple and fast CE/MS/MS method is presented for the study of labeling reaction with these new labeling reagents. These bromine-containing reagents simplify the MS/MS spectra of peptides: the charge of the derivatives increased the intensity of the corresponding ions, thus enhancing the sensitivity of the detection. The special isotopic distribution of bromine (the ^{79}Br and ^{81}Br isotope ratio is nearly one) facilitated to recognize the bromine-containing species. By eliminating the non-doubled peaks, clear and easily interpretable MS/MS spectra can be produced that contain only the labeled fragments. Owing to the isotopic distribution of bromine, the labeled peptides are easily recognized in the spectra of unknown peptide mixtures without recording and analyzing the spectra of the non-labeled samples. The separation of the labeled peptide and excess of labeling reagent was achieved with our home-built CE/MS interface that allowed the injection of a few nanoliters of sample thus this method can also be used in the case of extremely small sample quantities.

2. Experimental

All CE/MS/MS experiments were performed using a Finnigan TSQ 7000 triple quadrupole instrument (Finnigan MAT Ltd., San Jose, CA, USA) equipped with a Finnigan electro-spray source and with a home-built nanospray/CE ion source [2].

The nanospray tips were obtained by pulling borosilicate glass capillaries (1.2 mm O.D., 0.69 mm I.D., Clark Electromedical Instruments, Reading, England). Fused-silica capillaries (Polymicro Technologies, Phoenix, USA, I.D. 50 μm , O.D. 150 μm) were used as CE separation capillary. Capillaries were conditioned with 1 M NaOH, washed with deionized water by sucking with a water-jet pump and cut to 25 or 35 cm (for

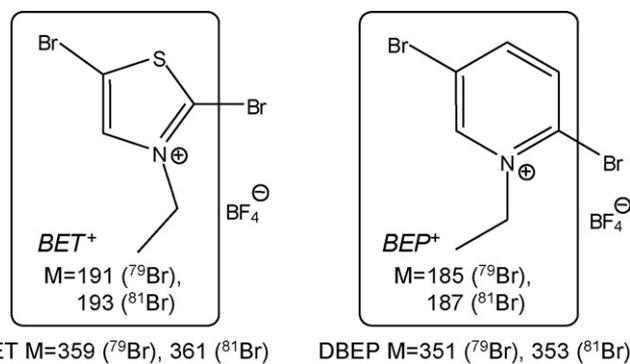


Fig. 1. The structures of 2,5-dibromo-1-ethyl-thiazolium (DBET) and 2,5-dibromo-1-ethyl-pyridinium (DBEP) tetrafluoroborates and their corresponding labels (BET^+ and BEP^+).

peptide mixture, Fig. 6) length. The capillary was filled with running buffer (methanol–water–acetic acid, 20:79:1, v/v/v) using a Hamilton glass syringe via a Teflon connector. The nanospray capillary tip had been adjusted to the proper position on the stage of the interface for electrospraying. The opposite end of the fused-silica capillary was immersed into the running buffer. The CE high-voltage power supply (Spellman, Plainview, USA) was connected to a platinum electrode which was inserted into the same Eppendorf tube. The ESI high-voltage power supply was joined directly to the gold-coated surface of the borosilicate capillary. +1.3 (nanospray needle) and +15 kV (CE capillary inlet) have been applied in these experiments [2]. The collision energy was varied between -20 and -40 eV to find the optimal value. The collision gas was argon and the pressure in the collision cell region was set to 0.27 ± 0.01 Pa.

Peptides (GPVYF-NH₂, DSCNYITR-NH₂, TSLRFSAK-NH₂, TSLGFSAK-NH₂, CNAEKNNHDGQG-NH₂, NYITR-NH₂, AQI) and labeling reagents 2,5-dibromo-1-ethyl-pyridinium (DBEP) and 2,5-dibromo-1-ethyl-thiazolium (DBET) tetrafluoroborate (Fig. 1) [14] have been synthesized in our laboratory. The labeling reaction conditions are as follows: 10 μL solution of peptides (water–acetonitrile = 1:1, v/v, 1 mg/mL) was mixed with the reagents (in acetonitrile 1 mg/mL) in a threefold molar excess, and then 40 μL 50 mM NH₄HCO₃ buffer was added. After 5–20 min (DBET or DBEP in case of C- and Y-containing peptides) or a few hours (DBEP in case of other peptides), samples of reaction mixtures were diluted to 0.01 mg/mL concentration with running buffer (methanol–water–acetic acid, 20:79:1, v/v/v) and injected electrokinetically into the capillary (+5 kV, 2–3 s).

3. Results and discussion

Labeling reagents were designed to react with the N-terminal amino group peptides, but ϵ -amino group of C-terminal Lys in tryptic fragments can be labeled as well. We wanted to increase the selectivity of the labeling reaction; therefore, first we optimized the conditions of the reaction. Two peptides were studied, containing only free N-terminal amino group (GPVYF-NH₂) and lysin (TSLGFSAK-NH₂). Because a threefold reagent was used, its excess should be separated from the labeled peptide

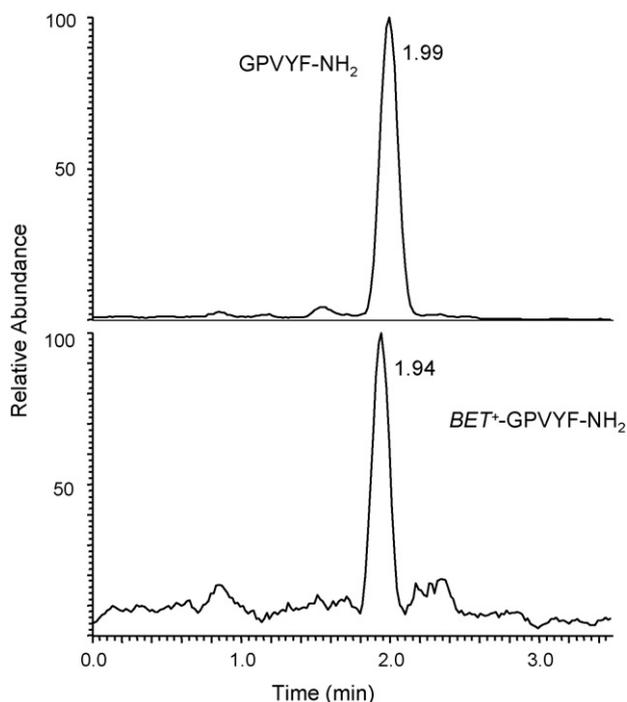


Fig. 2. CZE-MS of GPVYF-NH₂ and BET⁺-GPVYF-NH₂ peptides (N-terminal labels are shown in italics; length of the capillary: 20 cm; running buffer: methanol:water:acetic acid, 20:79:1, v/v/v; +15 kV to the injection end of the column and +1.3 kV to the nanospray needle were applied; injection: electrokinetically, +5 kV, 2–3 s).

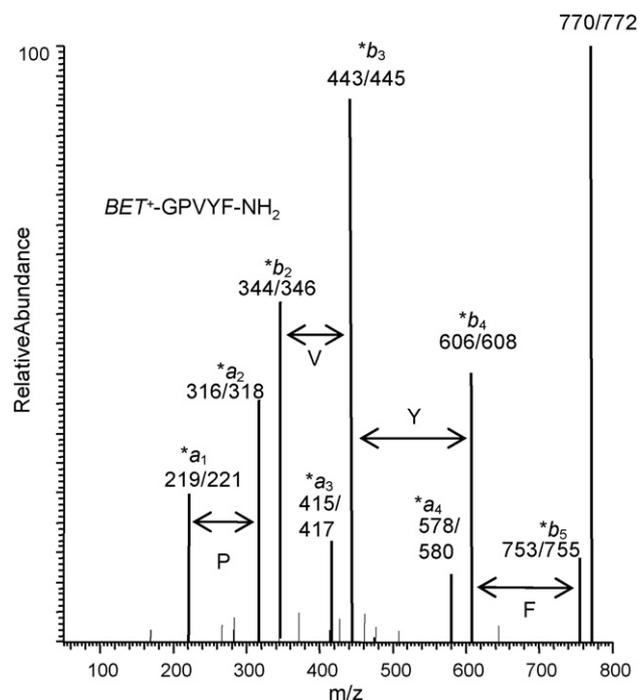


Fig. 3. MS/MS spectrum of labeled peptide BET⁺-GPVYF-NH₂ (N-terminal labels are shown in italics; thick lines indicate the double, bromine-containing peaks).

before the MS analysis. The resulting CE/MS electropherograms of the labeling reaction between GPVYF-NH₂ and DBET can be seen in Fig. 2. There is only a small difference in the migration time of the labeled and the unlabeled peptides proving that the free N-terminal amino group participated in the reaction. The MS/MS spectra of the 770 and 772 *m/z* ions (peptides containing ⁷⁹Br and ⁸¹Br isotopes) were recorded independently and the sum of the spectra was analyzed (Fig. 3). If we take into consideration only the double peaks, the spectrum can be interpreted easily. A series of **b* type ions (asterisks denote charged derivatives according to Roth et al. [4]; the detailed sequence and the possible fragmentation of labeled GPVYF-NH₂ and the

nomenclature of ions can be seen in Fig. 4) can be observed in the spectrum starting from 753/755 *m/z* (NH₃ loss of C-terminal amide) to 344/346 *m/z*. The mass differences of these ions correspond to the masses of amino acid residues F, Y and V. From 578/580 *m/z* to 219/221 *m/z*, other ions can be detected (series of **a* ions), where the mass differences correspond to Y, V, and P amino acids. The 219/221 *m/z* ion belongs to the **a* ion of labeled glycine. These results show that the label BET⁺ can be found on the N-terminal amino group rendering the spectrum interpretation easier. DBET was not successfully used for labeling peptides that contained free carboxylic group under used conditions with small reagent excess, but esterification of carboxyl groups could solve this problem.

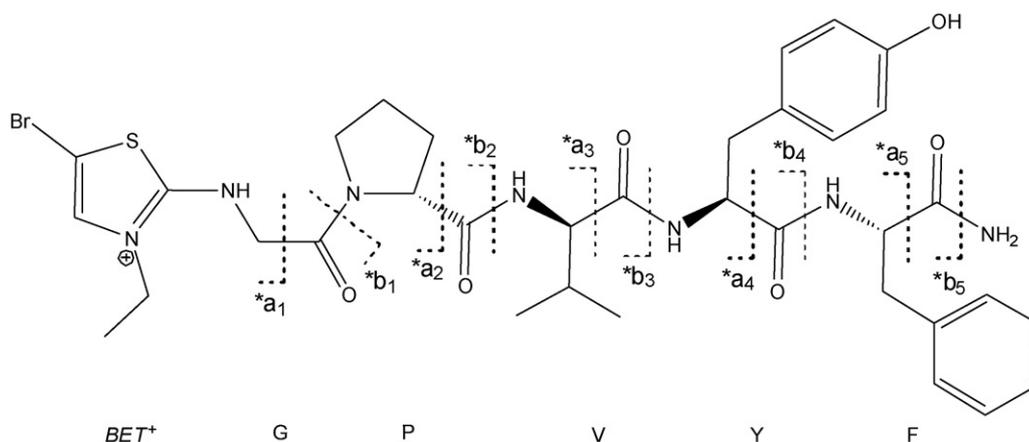


Fig. 4. Detailed sequence and the possible fragmentation of labeled peptide (BET⁺)GPVYF-NH₂ and the nomenclature of ions [4].

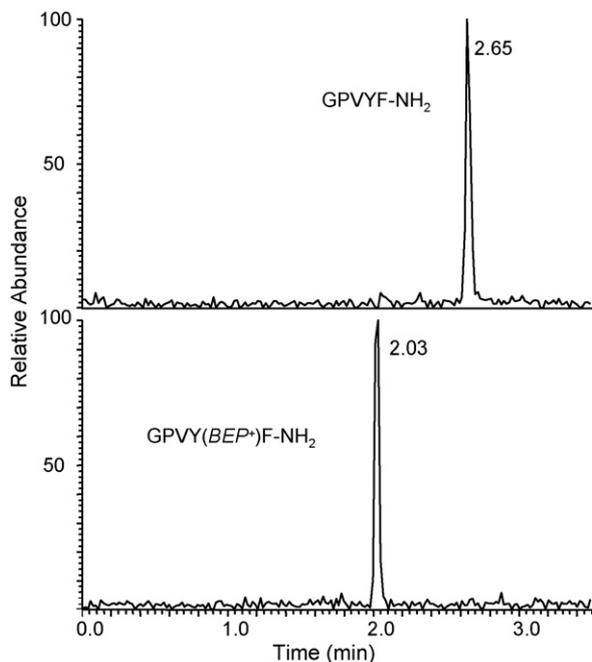


Fig. 5. CZE-MS analysis of GPVYF-NH₂ and GPVY(BEP⁺)F-NH₂ peptides (labels on Tyr are shown in parentheses in italics; length of the capillary: 25 cm; running buffer: methanol:water:acetic acid, 20:79:1, v/v/v; +15 kV to the injection end of the column and +1.3 kV to the nanospray needle were applied; injection: electrokinetically, +5 kV, 2–3 s).

A different behavior of the DBET can be observed in the case of TSLGFSAK-NH₂ where the peptide contains two amino groups. Almost a complete series of *y ions (from now on only the ⁸¹Br-containing derivatives were considered: 810, 697, 640, 493, 406, 335 *m/z*) could be found in the MS/MS spectrum indicating that DBET selectively reacts only with the ε-amino group in the side chain of Lys. Whereas we have a selective method for the introduction of a permanent positive charge to the Lys ε-amino group; we were interested whether this labeling decreases the difference in the intensity of Arg- and Lys-containing tryptic fragments. Thus, we have studied the labeled peptide TSLRFSAK-NH₂ containing both basic amino acids. In this case the labeled lysine enhances the intensity of C-terminal fragments by the permanent charge and also simplifies the sequence determination by unambiguous identification of bromine-containing fragments.

The electropherograms of DBEP-labeled and unlabeled GPVYF-NH₂ (Fig. 5) show quite a large difference in the migration time compared to the DBET-labeled and unlabeled peptides, indicating a difference in the charge of the labeled and original peptides. All peptides that we used in the experiments contain at least one amino group which is positively charged at the pH value of the analysis. Thus if a charged reagent bonds to the protonated amino group, the charge of the molecule does not change; hence the derivatization probably has no significant effect on the migration time. In contrast, by labeling tyrosine-OH group that is uncharged originally but gains charge due to the derivatization, a shorter migration time can be expected. The CE/MS of different (BEP⁺)-labeled peptides (Fig. 6) also supports this expectation. In the case of NYITR-NH₂ where Tyr is labeled,

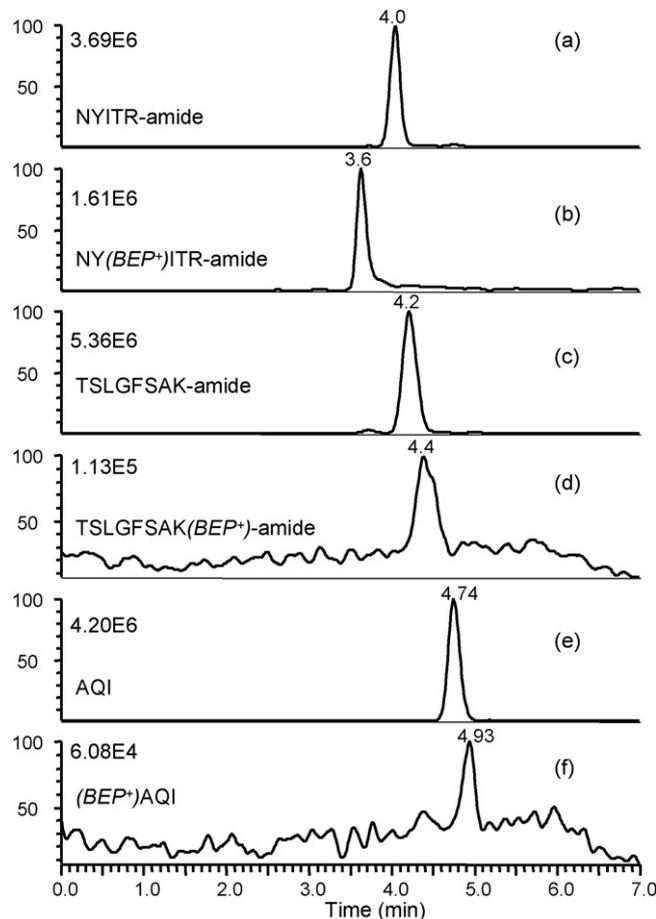


Fig. 6. CZE-MS analysis of mixture of (BEP⁺) labeled and unlabeled NYITR-NH₂, TSLGFSAK-NH₂ and AQI (labels are shown in parentheses in italics; length of the capillary: 35 cm; running buffer: methanol:water:acetic acid, 20:79:1, v/v/v; +15 kV to the injection end of the column and +1.3 kV to the nanospray needle were applied; injection: electrokinetically, +5 kV, 2–3 s).

the derivatized peptide migrates faster (Fig. 6, panel a and b). In other cases, where the lysine ε-amino (TSLGFSAK-NH₂, Fig. 6, panel c and d) or α-amino group is labeled (AQI, Fig. 6 panel e and f, the position of labeling will be proved later), there are no remarkable differences in the migration times: the labeled peptides migrate even slower. Thus, the direction of shifting in the migration times of labeled and unlabeled peptides would help in determining the position of labeling; hence it can be a useful support in elucidating their MS/MS spectra.

The MS/MS (Fig. 7) spectrum recorded by following the method described above shows different fragmentation pattern. In this spectrum an ion with 291/293 *m/z* (containing ⁷⁹Br and ⁸¹Br) appears with highest abundance among the Br-containing ions and no other labeled ions with lower mass could be found. Below 291 *m/z*, several non-doubled ions can be found, indicating that the N-terminal amino group is free (254 and 155 *m/z* are *b* ions of GPV and GP, and also the *a* ions of them can be found: 226 and 127 *m/z*). Considering the observations, the ion with 291 *m/z* can be an internal fragment of the labeled tyrosine. This assumption is supported by two other bromine-containing ions: 438 and 390 *m/z* ions corresponding to the masses of YF and VY fragments. On the basis of these results, DBEP labels the phenol

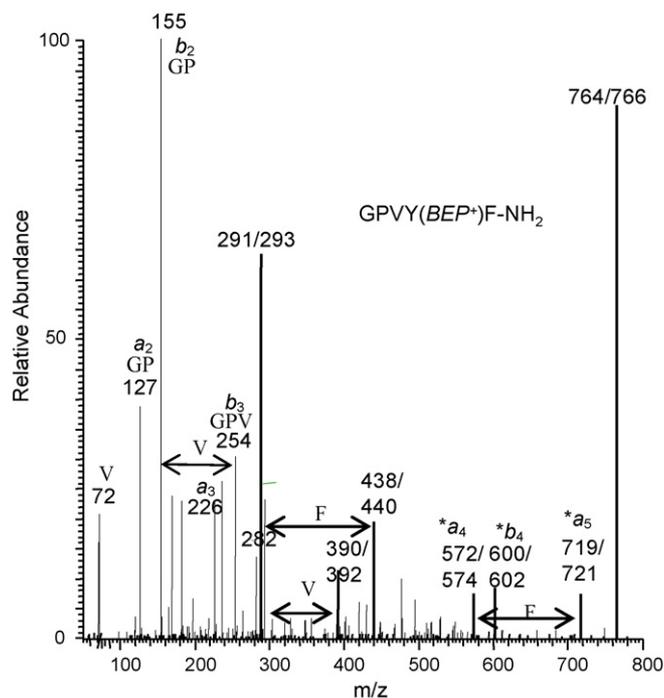


Fig. 7. MS/MS spectrum of labeled peptide GPVY(BEP⁺)F-NH₂ (labels on Tyr are shown in parentheses in italics; thick lines indicate the double, bromine-containing peaks).

group of tyrosine rather than its free N-terminal amino group. In order to check the previous idea, another Tyr-containing peptide (NYITR-NH₂) was allowed to react with DBEP. Bromine-containing ions (291/293 *m/z*) appeared again in the MS/MS spectrum together with a 70 *m/z* ion (immonium ion of N) and a series of **b* ions (832, 676, 576, 463 *m/z* corresponding to labeled NYITR, NYIT, NYI, NY sequences) along with a series of **a* ions, indicating that the label can be found on the tyrosine residue. Based on these phenomena, 291/293 *m/z* ion pair can be easily used (*e.g.* with precursor ion scan) as a diagnostic ion for recognizing tyrosine-containing peptides with enhanced intensity and selectivity. To clarify the nature of DBEP reagent, it was allowed to react with peptides containing aliphatic hydroxyl and thiol groups. The MS/MS spectra of the labeled peptides DSCNYITR-NH₂ and CNAEKNNHDGQG-NH₂ show that the cysteine thiol group reacts with DBEP: an intense bromine-containing peak appeared (218/220 *m/z*) which can be assigned as the result of side-chain fragmentation product of labeled cysteine. In the spectrum of labeled DSCNYITR-NH₂, no peak was found, proving the presence of labeled tyrosine or serine. If the peptides contain neither Cys nor Tyr amino acids DBEP reacts with their free amino groups but the reaction takes a few hours. However, being less reactive it is compatible with carboxyl groups (see the results of AQI peptide below). In order to examine the selectivity of the reaction two additional peptides were allowed to react with DBEP. The peptide AQI contains only an N-terminal amino group, and peptide TSLGFSAK-NH₂ has both N-terminal and lysine ϵ -amino groups. As expected, AQI peptide was labeled on the N-terminal amino group, and series of **b* + H₂O ions, 516, 403, 274 *m/z*, correspond to labeled AQI, AQ, A and *a*^{*} ions at 357 *m/z* (AQ) and 229 *m/z* (labeled A).

There was no reaction between the carboxyl group and DBEP. In contrast to our previous results, the MS/MS spectrum of peptide TSLGFSAK-NH₂ shows **y* series (807, 693, 636, 489 *m/z* corresponding to labeled LGFSAK, GFSAK, FSAK and SAK, respectively) together with a series of unlabeled *b* ions (302, 189 *m/z*) and *a* ions (284, 171 *m/z*) that can be identified as unlabeled N-terminal part of the sequence (TSL and TS). These results corroborate that DBEP selectively labeled the ϵ -amino group of lysine.

4. Conclusion

In conclusion, our new bromine-containing positively charged reagents 2,5-dibromo-1-ethyl-thiazolium (DBET) and 2,5-bromo-1-ethyl-pyridinium (DBEP) salts improved the interpretation of MS/MS spectra of peptides due to the isotope distribution of bromine. Labeling reactions were analyzed simply and fast with the CE/MS/MS method. Tyr- and Cys-labeled peptides migrated faster because of the introduction of additional positive charge onto the peptides; thus the direction of shifting in the migration times of labeled and unlabeled peptide would help in interpreting their MS/MS spectra. MS/MS spectra from the peaks of electropherograms gave information on the position of the label in peptides. Labeling reactions are selective due to the large differences in the reactivity of different functional groups of the peptide. DBET reacts in the order: ϵ - >> α -amino group >> hydroxyl group of Tyr, while DBEP reacts preferably with thiol group of Cys >> hydroxyl group of Tyr. DBEP labels amino groups in a much slower reaction than DBET but with the same selectivity. The presence of a Tyr or Cys amino acid in a peptide can be easily determined by characteristic ion pairs 291/293 and 218/220 *m/z*, respectively.

Acknowledgments

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