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Matrix-assisted laser desorption/ionization mass spectrometry peptide sequencing utilizing selective N-terminal bromoacetylation

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ABSTRACT

In tandem mass spectrometric peptide sequencing, simplifying the mass spectrum is often desirable. The b-series ions were distinguished from the y-series ions in the MALDI TOF-TOF spectra by incorporating a bromine-tag to the N-terminal amino group through rapid and selective acetylation using bromoacetic anhydride without blocking the lysine and tyrosine residues. The 51:49 ratio of Br-79 and Br-81 isotopes facilitated identification of ions carrying the tag. With the Br-tag in the b-series ions, N-terminal sequencing of tryptic peptides from hemoglobin as well as model peptides was straightforward. When the b-ions were low in intensity, ions without the Br-tag were identified as y-ions and used for sequencing.

In both matrix-assisted laser desorption/ionization (MALDI)[1] [1,2], and electrospray ionization (ESI) [3] methods, tandem mass spectrometry (MS/MS) is widely used for structural characterization [4], amino acid sequencing in particular. In tandem MS, multiple ions are produced leading to complex mass spectra. To simplify spectral analysis, the N-terminal amino group can be chemically modified either to enhance or to suppress the b- or y-ion series. Fragmentation by electron transfer dissociation (ETD) [5] or electron capture dissociation (ECD) [6,7] leads to c- and z-series ions. These methods are used in the ESI mode and are not applicable to MALDI MS. Sequencing of peptides using w-, y-, and x-ions generated by 157 nm laser needs specialized instrumentation [8].

In chemically assisted fragmentation, the N-terminus is often sulfated to carry a negative charge so that the b-series ions are suppressed [9–12]. In this case, since tryptic peptides with C-terminal lysine or arginine are often used to take advantage of the strong y-ion peaks, guanidination of the C-terminal lysine is necessary to avoid sulfation of the C-terminus. Moreover, sulfation of the peptide decreases the intensity of the parent ion to be analyzed by tandem MS.

There are several N-terminal modification methods that do not involve suppression of b-ions [13–20]. We were interested in developing a chemical modification method for introducing a bromine-tag to the N-terminal amino group even at the expense of suppressed b-ions. The unique bromine isotopic ratio (50.69% Br-79, 49.31% Br-81) was utilized to identify cysteine, [21] or to monitor the reaction pathway of Br-containing compounds such as bromobenzene with proteins [22,23]. Bromine-isotope tag was also used in capillary electrophoresis detection by introducing positively charged compound to the N-terminal amino acid of a peptide [24]. The bromine-tag had been used for various applications [25–28]. We intended to selectively modify the N-terminus using bromoacetic anhydride. Bromoacetylation with bromoacetic anhydride is pH dependent and thus can be carried out without protecting the lysine residue at pH around 6 [29]. This approach had been used in the past to introduce a bromoacetyl moiety to peptides and to use it as a linker to DNA or polymers [29,30]. The unique isotopic abundance of bromine was expected to produce a unique isotopic pattern and facilitate selection of the b-ion peaks in the presence of strong y-ion peaks in both MS and MS/MS spectra. In particular, in peptides of 1000–2000 Da molecular weight most useful in peptide analyses, the first and the third isotopic peaks would stand out making selection of the tagged ions straightforward. In this paper, we show that the bromoacetyl group can be selectively incorporated to the N-terminal amino acid in the presence of lysine residues and facilitate peptide sequencing using Br-tagged b-ions in the MS/MS analysis of model peptides and a peptide mixture generated by tryptic hydrolysis of a protein.

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[1] *Abbreviations used:* ECD, electron capture dissociation; ETD, electron transfer dissociation; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; CHCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid.

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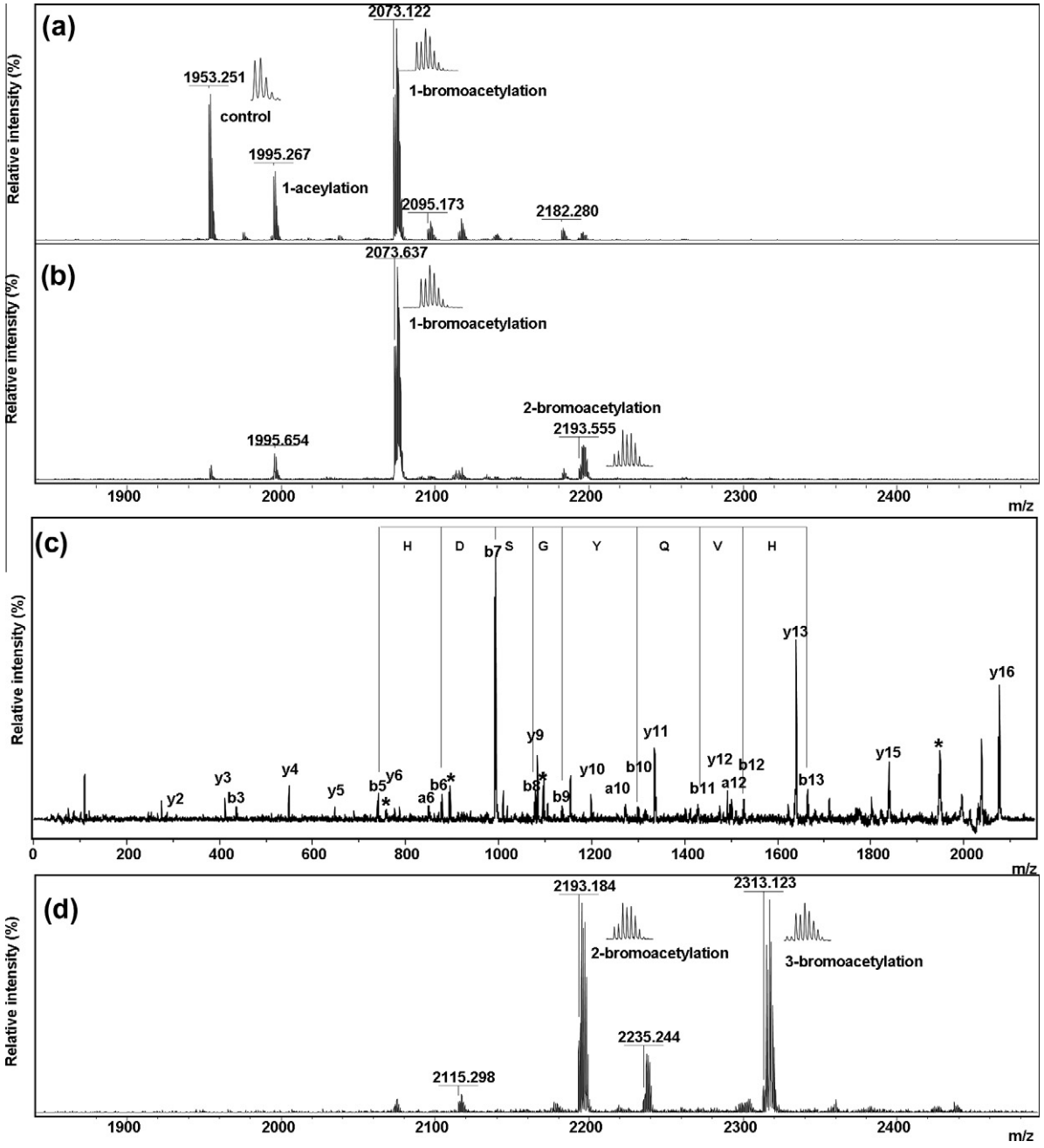

Fig.1. MALDI mass spectra showing pH dependence of bromoacetylation of DAEFRHDSGYQVHHQK in distilled water (a), 50 mM Hepes buffer, pH 7.0 (b), and 75 mM Tris buffer, pH 8.8 (d). Panel c shows the MS/MS spectrum from the 2073.637 peak in b.

Materials and methods

Materials

Bromoacetic acid, diisopropylcarbodiimide, model peptides and proteins, α-cyano-4-hydroxycinnamic acid (CHCA), and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, USA). Dichloromethane and acetonitrile were from Fischer (Pittsburgh, USA). Distilled water was prepared using the Milli-Q system from Millipore (Billerica, USA).

Preparation of bromoacetic anhydride

Bromoacetic anhydride was made by the published method [30]. The amount of 0.278 g of bromoacetic acid (2 mmol) was dissolved in 1 ml of dichloromethane in an Eppendorf tube and 157 μl of diisopropylcarbodiimide (1 mmol) was added. After mixing for 30 min using a vortex mixer, the mixture was filtered using a 0.45 μm filter (Millipore). When the solvent was removed using a Speed-Vac for 30 min, a light yellowish oily liquid of bromoacetic anhydride was obtained.

Bromoacetylation of model peptides

Model peptides were dissolved in 50 mM, pH 7.0, Hepes buffer to give 5 μM concentration. A 0.1% (v/v) bromoacetic anhydride dissolved in acetonitrile was kept cold in ice water at 0 °C, and 2 μl of the model peptide solution was mixed with 10 μl of the cold bromoacetic anhydride solution and left cold for 10 min. Then 8 μl water was added to terminate the reaction. After 5 min in ice

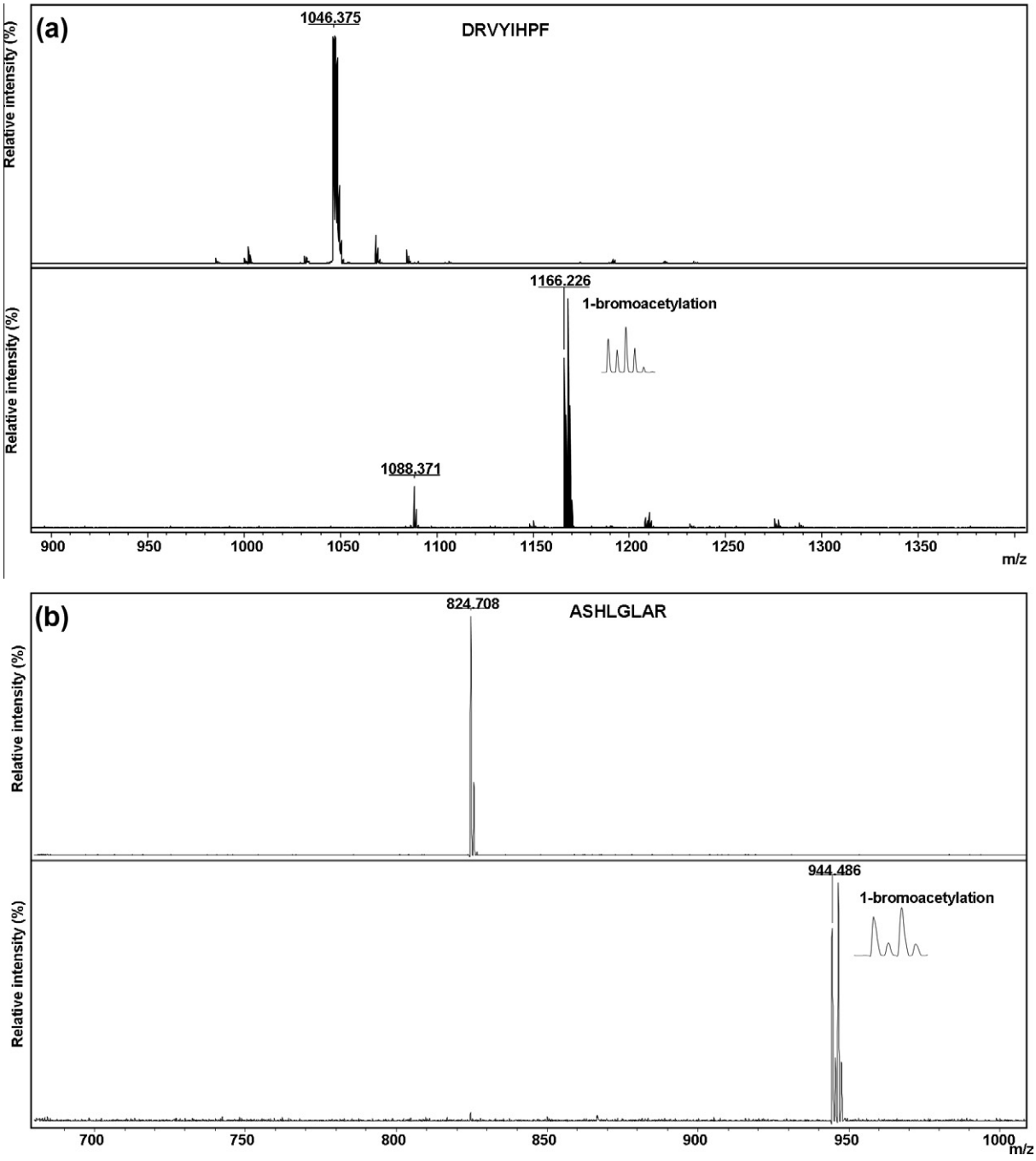

Fig.2. Bromoacetylation of various peptides at pH 7.0. (a) DRVYIFPH, (b) ASHLGLAR, (c) WHWLQLKPGQPMY, (d) FQ*p*SEEQQQTEDELQDK.

water, solvents were removed using SpeedVac and the remaining dried sample was dissolved in 1 μl aliquot of 0.1% TFA solution.

Bromoacetylation of tryptic peptides

Several milligrams of bovine hemoglobin or bovine serum albumin was dissolved in 1 ml 50 mM ammonium bicarbonate buffer, heat denatured, and digested with 7–10% (w/w) trypsin for 6 h. Tryptic peptides produced were dissolved in 50 mM ammonium bicarbonate to 50 μM concentration and subjected to bromoacetylation as above for model peptides.

MADLI-TOF/TOF MS

The amount of 700 μl acetonitrile containing 0.1% TFA was mixed with 300 μl distilled water also containing 0.1% TFA and 10 mg CHCA was dissolved in the mixture. Then the solution was saturated with nitrilotriacetic acid and centrifuged. One microliter of the matrix solution was mixed with 1 μl of the sample solution, loaded on the sample plate, and dried. MALDI TOF/TOF MS was carried out using Autoflex (Bruker Daltonics, Bremen, Germany). Mass spectra were obtained in the positive reflector mode. Peptide standard from Bruker was used for mass calibration. MS/MS spectra were obtained by averaging 1000 shots.

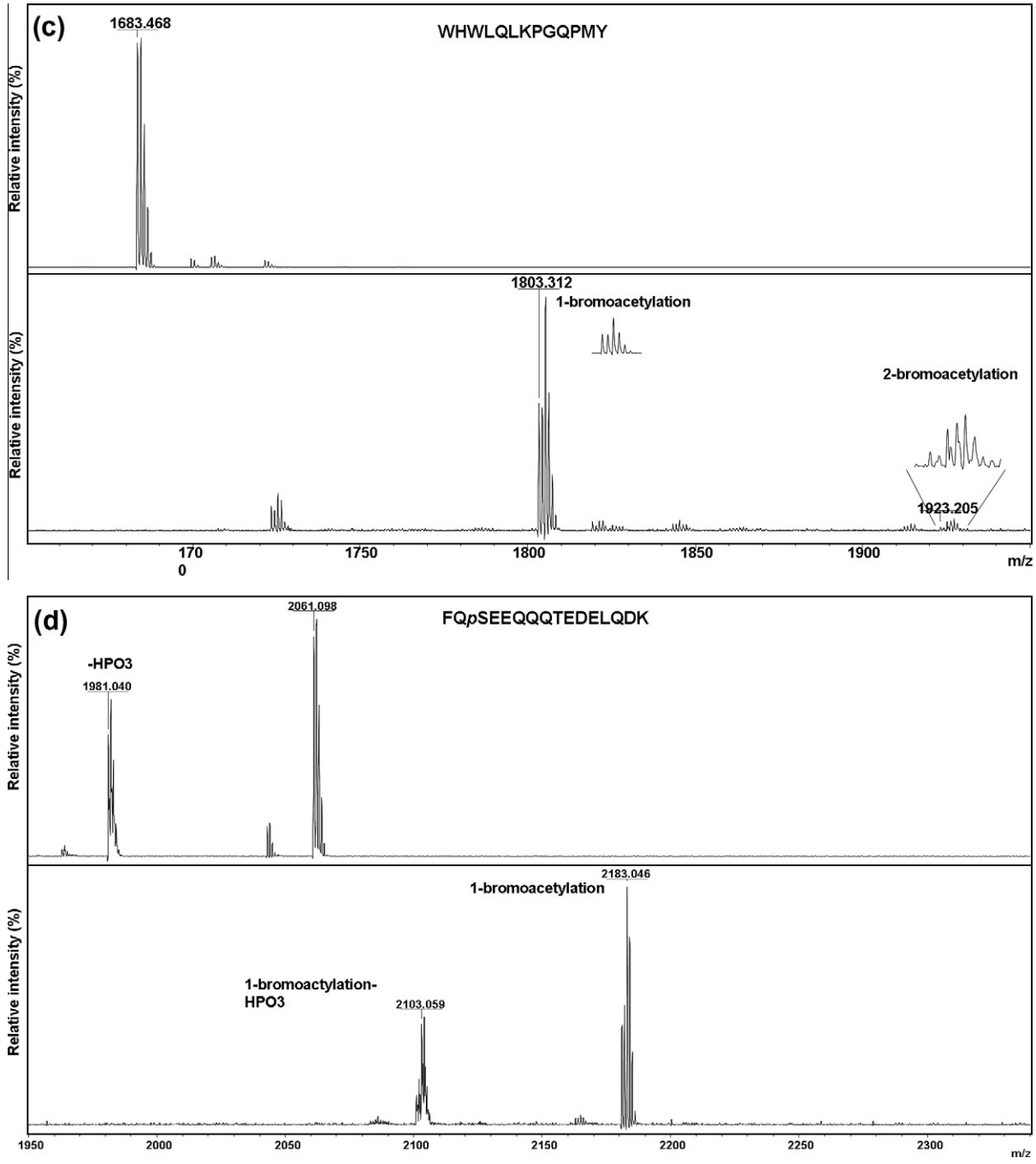

Fig.2 (*continued*)

Results and discussion

Bromoacetylation

Acetylation is known to take place at lysine and tyrosine residues as well as at the N-terminus in a pH-dependent manner [29]. The reaction is slow at low pH and thus site specific, whereas it takes place at all sites at high pH. Therefore, it is important to find reaction conditions for selective bromoacetylation at the N-terminus.

Our preliminary test showed that bromoacetylation yield is low when water is present in excess over acetonitrile. It is probably because hydrolysis of bromoacetic anhydride is faster than bromoacetylation. Therefore, the reaction was carried out in excess acetonitrile.

Under optimal conditions, bromoacetylation by bromoacetic anhydride was immediate at room temperature and thus control of lysine and tyrosine bromoacetylation was difficult. After 5–10 min at 0 °C, singly bromoacetylated peptide was obtained as a major reaction product at optimal pH.

Fig. 1 shows the pH dependence of bromoacetylation, carried out at 0 °C for 10 min, of a model peptide containing lysine and tyrosine (DAEFRHDSGYQVHHQK). Bromoacetylation was incomplete when 2 μl of the peptide solution in deionized water was mixed with 10 μl of bromoacetic anhydride solution in acetonitrile. The bromoacetylation peak at 2073.122 with a characteristic bromine isotopic pattern was the strongest; however, the control peptide peak at 1953.251 without the bromine pattern was also strong (Fig. 1(a)). The pH of the reaction mixture was below 5 due to hydrolysis of bromoacetic anhydride to bromoacetic acid. When

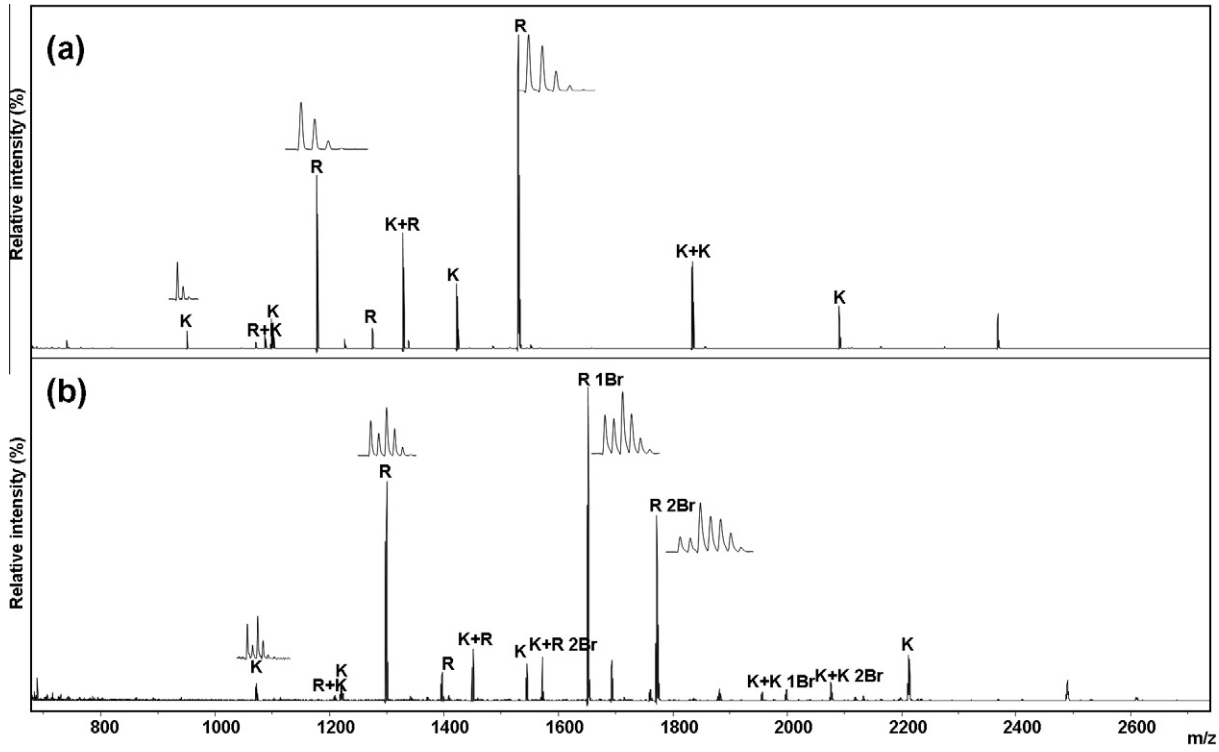

Fig.3. MALDI mass spectra from bovine hemoglobin tryptic peptides before (a) and after (b) bromoacetylation showing 120 Da mass shift.

Table 1
Hemoglobin tryptic peptides carrying bromoacetylation tag. Most lysine-containing peptides are one bromoacetylated.

Start	–	End	Observed	Sequence	1 Br	2Br	MS/MS
76b	–	81b	740.3807	HLDDL**K**	o		o
8b	–	16b	950.4689	AAVTAFWG**K**	o		o
32a	–	40a	1071.5255	MFLSFPTT**K**	o		o
91a	–	99a	1087.5941	LRVDPVNF**K**	o		
95b	–	103b	1098.4916	LHVDPENF**K**	o		
19b	–	29b	1101.6016	VDEVGGEALGR	o		
132b	–	143b	1177.6766	VVAGVANALAHR	o		o
30b	–	39b	1274.7255	LLVVYPWTQR	o		o
17b	–	29b	1328.7214	V**K**VDEVGGEALGR	o		o
120b	–	131b	1422.7166	EFTPVLQADFQ**K**	o		o
17a	–	31a	1529.7492	VGGHAAEYGAEALER	o	o	o
41a	–	56a	1833.9187	TYFPHFDLSHGSAQV**K**	o	o	o
40b	–	58b	2089.9834	FFESFGDLSTADAVMNNP**K**	o		
69a	–	90a	2367.2246	AVEHLDDLPGALSELSDLHAH**K**	o	o	

the peptide dissolved in 50 mM Hepes, pH 7.0, buffer was used, the bromoacetylation peak was the strongest and the doubly bromoacetylated peptide ion showed a small peak at 2193.555, while the control peptide peak almost disappeared (Fig. 1(b)). MS/MS analysis of the mono-bromoacetylated peptide ion at 2073.637 showed b-series ions consistent with tagging at the N-terminus only (Fig. 1(c)), indicating that bromoacetylation at the N-terminus was strongly favored. At pH 8.8 in 75 mM Tris buffer, doubly and triply bromoacetylated peptides were dominant, suggesting that both lysine and tyrosine residues as well as the N-terminal amino group were tagged. Both doubly and triply tagged peptides were observed when the reaction was carried out at room temperature for 5 min at pH 7. These results show that selective bromoacetylation at the N-terminus can be achieved by pH and temperature control.

To establish that the selected reaction conditions general apply, bromoacetylation of 10 additional peptides was tested. Fig. 2 shows that bromoacetylation for 10 min at 0 °C and pH 7.0 produced mono-bromoacetylation product in a peptide with one tyrosine (DRVYIFPH, Fig. 2(a)), in a peptide with one lysine in the middle and a C-terminal tyrosine (WHWLQLKPGQPMY, Fig. 2(c)), and in a phosphopeptide with a C-terminal lysine (FQ*p*SEEQQQTEDELQDK, Fig. 2(d)). They all showed a mono-bromoacetylation peak as the major product. Also several peptides with different N-terminal amino acids were synthesized and tested for the influence of the N-terminal amino acids on tagging efficiency. Some variation was observed; however, single N-terminal bromoactylation was predominant as verified by MS/MS analyses (supplementary material).

Mono-bromoacetylation was also demonstrated using a mixture of tryptic peptides. If the N-terminal amino groups of all peptides are preferentially tagged before lysine residues, a mass shift of 119.92 Da should be observed in all tryptic peptides. Fig. 3 shows mass spectra from control (Fig. 3(a)) and bromoacetylated (Fig. 3(b)) bovine hemoglobin tryptic peptides. Most hemoglobin tryptic peptides have C-terminal lysine. Yet practically all

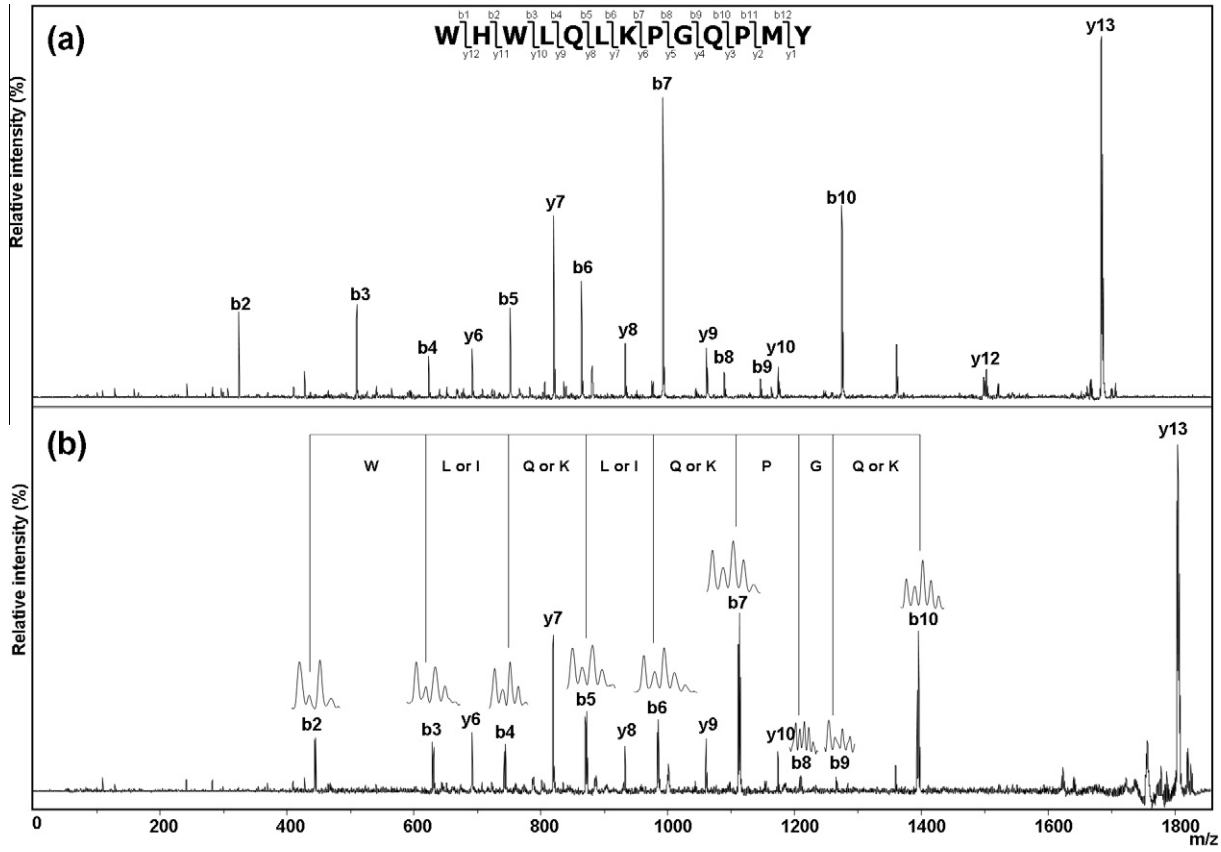

Fig.4. MALDI mass spectra from WHWLQLKPGQPMY before (a) and after (b) bromoacetylation. Br-tag was observed only in the b-series ions.

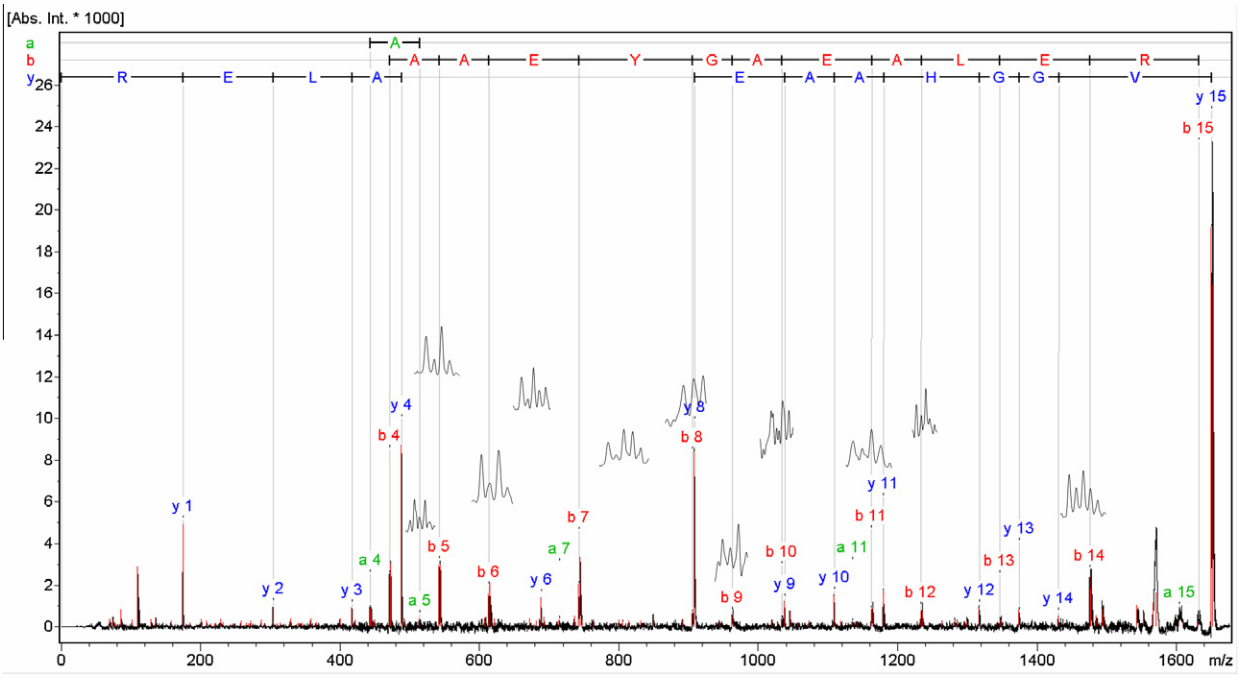

Fig.5. MS/MS spectrum from one of hemoglobin tryptic peptides.

tryptic peptides were singly tagged except the chain A 17-31 peptide which showed double tagging as well. Fig. 3(b) shows little unmodified peptides without the Br-tag, except a small A 17-31 peptide peak. The result indicates that bromoacetylation is almost complete regardless of the N-terminal amino acid. The N-terminal amino acids of hemoglobin tryptic peptides that left no observable unmodified peptide signal include histidine, alanine, methionine, leucine, valine, glutamic acid, threonine, and phenylalanine as

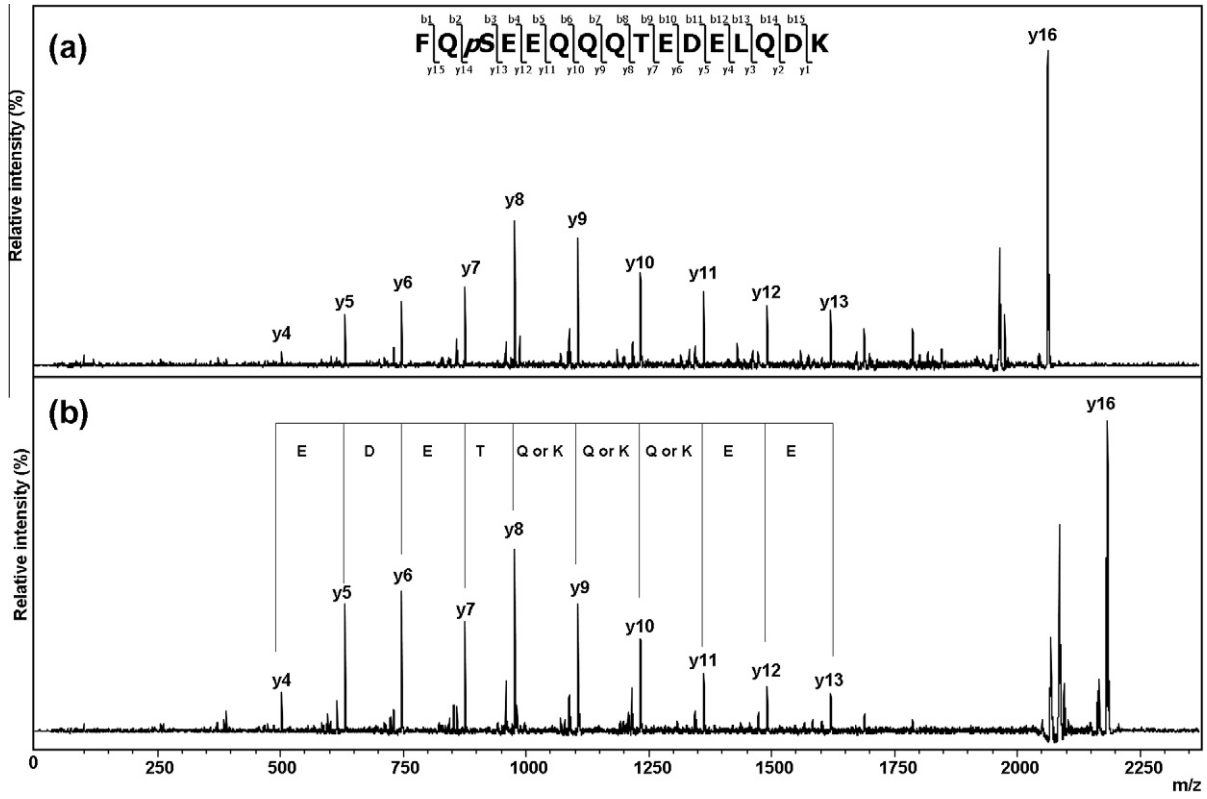

Fig.6. MS/MS spectra from FQpSEEQQQTEDELQQK before (a) and after (b) bromoacetylation showing y-ions only.

summarized in Table 1. Most of the peptides were singly tagged, while 3 of the 14 peptides were doubly tagged.

It must be pointed out that sensitivity of detection is compromised by bromoacetylation of the basic N-terminal amine group. When tryptic peptides equivalent to 400 fmol hemoglobin in 1 μl were loaded on the plate, the strongest peak with the Br-tag was observed with S/N of about 70, which was enough to perform MS/MS experiment. This corresponded to about 3-fold reduction in signal intensity upon bromoacetylation. We believe that the advantages of the Br-tag could offset the reduced signal in many circumstances, particularly if more powerful mass spectrometers are used.

MS/MS analysis of b-series ions with Br-tag

Since the Br-tag is introduced to the N-terminus, the Br signature is expected in the b-series ions. Fig. 4 shows the MS/MS spectra from a model peptide (WHWLQLKPGQPMY) before and after bromoacetylation. Distinction between b- and y-series is not straightforward without the Br-tag. In de novo sequencing, the b- and y-ion pair is searched based on the fact that their sum should match the mother ion mass. In that case, which is b-ion and which is y-ion is not straightforward. The assignment is possible upon analysis of the entire spectrum.

With Br-tag, assignment of the b- and y-series ions is straightforward. The peaks in Fig. 4(b) carrying the Br-tag could be easily assigned as b2, b3, b4, b5, b6, b7, b8, b9, and b10 ions. Moreover, peaks without the Br-tag could be assigned as y-ions, which were also observed without bromoacetylation (Fig. 4(a)).

Admittedly, the Br-tag at the N-terminus could lead to a-ions as well as b-ions complicating the analysis. Another concern about using the Br-tag is that some of the b-ions may not be detected from tryptic peptides, where the y-ions are stronger due to the lysine and arginine residues at the C-terminus.

Fig. 5 shows an example of partial sequencing using the b-ions and full sequencing using both b- and y-ions. When one of the tryptic peptides from bovine hemoglobin was subjected to MS/MS analysis, both b- and y-ions were observed in about equal abundance. Interestingly, the y-ions were not necessarily stronger. Anyhow, the Br-tag was particularly useful when identification of the peaks was difficult due to the low signal intensity. MS/MS data for other peptides are provided in the supplementary material. These results show that the b-ions obtained after bromoacetylation can be used for de novo sequencing of peptides.

MS/MS analysis without b-series ions

In an extreme case, the b-ions could be completely suppressed by the y-ions and not observed at all. In that case, most of the observed peaks will be from y-ions and carry no Br-signature. Fig. 6 shows mass spectra from a beta-casein phosphopeptide (FQpSEEQQQTEDELQDK). The negatively charged phosphate group is near the N-terminus. Moreover, there are many acidic amino acids in the peptide. Therefore, the b-ion peaks with negatively charged groups are expected to be low in intensity in the positive ion mode in the presence of y-ions with positively charged, basic C-terminal residues. In fact, all the fragment ions observed before and after bromoacetylation were identical except the mass-shifted y16 mother ion. Interpretation of the common ions as y-ions enabled sequencing of nine consecutive residues in the phosphopeptide.

Conclusion

The N-terminal amino group of peptides can be selectively bromoacetylated by a simple reaction with bromoacetic anhydride for 10 min at 0 °C and pH 7.0. MS/MS analysis of bromoacetylated peptide shows Br-tag in the b-ions, but not in y-ions. This distinction

between b- and y-ions facilitates de novo sequencing of peptides whether the b-ions with the Br-tag were observed or not.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.11.022.

References

[1] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yohida, Protein and polymer analyses up to m/z 100, 000 by laser ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 2 (1988) 151–153.
[2] M. Karas, F. Hillenkamp, Laser desorption Ionization of proteins with molecular masses exceeding 10000 daltons, Anal. Chem. 60 (1988) 2299–2301.
[3] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass-spectrometry of large biomolecules, Science 246 (1989) 64–71.
[4] A.K. Shukla, J.H. Futrell, Tandem mass spectrometry: dissociation of ions by collisional activation, J. Mass Spectrom. 35 (2000) 1069–1090.
[5] J.E.P. Syka, J.J. Coon, M.J. Schroeder, J. Shabanowitz, D.F. Hunt, Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry, in: Proc. Natl. Acad. Sci. USA, 101 (2004), pp. 9528–9533.
[6] R.A. Zubarev, N.L. Kelleher, F.W. McLafferty, Electron capture dissociation of multiply charged protein cations. A nonergodic process, J. Am. Chem. Soc. 120 (1998) 3265–3266.
[7] S. Lee, G. Chung, J. Kim, H.B. Oh, Electron capture dissociation mass spectrometry of peptide cations containing a lysine homologue: a mobile proton model for explaining the observation of b-type product ions, Rapid Commun. Mass Spectrom. 20 (2006) 3167–3175.
[8] T.Y. Kim, J.C. Schwartz, J.P. Reilly, Development of a linear ion trap/orthogonal-time-of-flight mass spectrometer for time-dependent observation of product ions by ultraviolet photodissociation of peptide ions, Anal. Chem. 81 (2009) 8809–8817.
[9] T. Keough, R.S. Youngquist, M.P. Lacey, A method for high-sensitivity peptide sequencing using post source decay matrix-assisted laser desorption ionization mass spectrometry, in: Proc. Natl. Acad. Sci. USA, 96 (1999), pp. 7131–7136.
[10] Y.H. Lee, H. Han, S.B. Chang, S.W. Lee, Isotope-coded N-terminal sulfonation of peptides allows quantitative proteomic analysis with increased de novo peptide sequencing capability, Rapid Commun. Mass Spectrom. 18 (2004) 3019–3027.
[11] Y.H. Lee, M.S. Kim, W.S. Choie, H.K. Min, S.W. Lee, Highly informative proteome analysis by combining improved N-terminal sulfonation for de novo peptide sequencing and online capillary reverse-phase liquid chromatography/tandem mass spectrometry, Proteomics 4 (2004) 1684–1694.
[12] I.R. Leon, A.G.C. Neves-Ferreira, R.H. Valente, E.M. Mota, H.L. Lenzi, J. Perales, Improved protein identification efficiency by mass spectrometry using N-terminal chemical derivatization of peptides from Angiostrongylus costaricensis, A nematode with unknown genome, J. Mass Spectrom. 42 (2007) 1363–1374.
[13] M.L. Hennrich, S. Mohammed, A.F.M. Altelaar, A.J.R. Heck, Dimethyl isotope labeling assisted de novo peptide sequencing, J. Am. Soc. Mass Spectrom. 21 (2010) 1957–1965.
[14] R.L. Beardsley, L.A. Sharon, J.P. Reilly, Peptide de novo sequencing facilitated by a dual-labeling strategy, Anal. Chem. 77 (2005) 6300–6309.
[15] Q. Fu, L.J. Li, De novo sequencing of neuropeptides using reductive isotopic methylation and investigation of ESI QTOF MS/MS fragmentation pattern of neuropeptides with N-terminal dimethylation, Anal. Chem. 77 (2005) 7783–7795.
[16] T.Y. Samgina, S.V. Kovalev, V.A. Gorshkov, K.A. Artemenko, N.B. Poljakov, A.T. Lebedev, N-terminal tagging strategy for de novo sequencing of short peptides by ESI-MS/MS and MALDI-MS/MS, J. Am. Soc. Mass Spectrom. 21 (2010) 104–111.
[17] M. Yamaguchi, T. Nakazawa, H. Kuyama, T. Obama, E. Ando, T. Okamura, N. Ueyama, S. Norioka, High-throughput method for N-terminal sequencing of proteins by MALDI mass spectrometry, Anal. Chem. 77 (2005) 645–651.
[18] M. Yamaguchi, D. Nakayama, K. Shima, H. Kuyama, E. Ando, T. Okamura, N. Ueyama, T. Nakazawa, S. Norioka, O. Nishimura, S. Tsunasawa, Selective isolation of N-terminal peptides from proteins and their de novo sequencing by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry without regard to unblocking or blocking of N-terminal amino acids, Rapid Commun. Mass Spectrom. 22 (2008) 3313–3319.
[19] F. Wang, H. Fu, Y.Y. Jiang, Y.F. Zhao, A picomole-scale method for rapid peptide sequencing through convenient and efficient N-terminal phosphorylation and electrospray ionization mass spectrometry, J. Am. Soc. Mass Spectrom. 17 (2006) 995–999.
[20] H. Kuyama, K. Sonomura, O. Nishimura, S. Tsunasawa, A method for N-terminal de novo sequence analysis of proteins by matrix-assisted laser desorption/ionization mass spectrometry, Anal. Biochem. 380 (2008) 291–296.
[21] T.V. Minaeva, E.N. Danilovtseva, V.V. Annenkov, A.V. Novikov, A.L. Vereshchagin, M.A. Grachev, A new bromine-containing reagent for cysteine modification, Russ. J. Bioorg. Chem. 33 (2007) 549–553.
[22] W.M. Yue, Y.M. Koen, T.D. Williams, R.P. Hanzlik, Use of isotopic signatures for mass spectral detection of protein adduction by chemically reactive metabolites of bromobenzene: studies with model proteins, Chem. Res. Toxicol. 18 (2005) 1748–1754.
[23] A. LeBlanc, T.C. Shiao, R. Roy, L. Sleno, Improved detection of reactive metabolites with a bromine-containing glutathione analog using mass defect and isotope pattern matching, Rapid Commun. Mass Spectrom. 24 (2010) 1241–1250.
[24] G. Ferenc, P. Padar, T. Janaky, Z. Szabo, G.K. Toth, L. Kovacs, Z. Kele, Capillary electrophoresis tandem mass spectrometry of bromine-containing charged derivatives of peptides, J. Chromatogr. A 1159 (2007) 119–124.
[25] M. Shin, H.J. Kim, Peptide C-terminal sequence analysis by MALDI-TOF MS utilizing EDC coupling with Br signature, Bull. Korean Chem. Soc. 32 (2011) 1183–1186.
[26] J.-S. Kim, M. Shin, J.-S. Song, S. An, H.-J. Kim, C-terminal de novo sequencing of peptides using oxazolone-based derivatization with bromine signature, Anal. Biochem. 419 (2011) 211–216.
[27] J.S. Kim, J. Kim, J.M. Oh, H.J. Kim, Tandem mass spectrometric method for definitive localization of phosphorylation sites using bromine signature, Anal. Biochem. 414 (2011) 294–296.
[28] J.-S. Kim, S.-U. Song, H.-J. Kim, Simultaneous identification of tyrosine phosphorylation and sulfation sites utilizing tyrosine-specific bromination, J. Am. Soc. Mass Spectrom. 22 (2011) 1916–1925.
[29] R. Wetzel, R. Halualani, J.T. Stults, C. Quan, A general method for highly selective cross-linking of unprotected polypeptides via pH-controlled modification of N-terminal alpha-amino groups, Bioconjugate Chem. 1 (1990) 114–122.
[30] M.M. Ma, A. Paredes, D. Bong, Intra- and intermembrane pairwise molecular recognition between synthetic hydrogen-bonding phospholipids, J. Am. Chem. Soc. 130 (2008) 14456–14458.