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Rapid and Efficient MALDI-TOF MS Peak Detection of 2-Nitrobenzenesulfonyl-Labeled Peptides Using the Combination of HPLC and an Automatic Spotting Apparatus

Tetsuo Iida,^{1*} Hiroki Kuyama,^{2*} Makoto Watanabe,¹ Chikako Toda,¹ Ei-ichi Matsuo,¹ Atsushi Kido,¹ Eiji Ando,¹ Susumu Tsunasawa,¹ and Osamu Nishimura^{1,2}

¹Life Science Laboratory, Shimadzu Corporation, Kyoto, Japan; ²Institute for Protein Research, Osaka University, Osaka, Japan

In this paper, we report MALDI-TOF MS analysis of 2-nitrobenzenesulfonyl (NBS) labeled peptides with the powerful aid of an LC-automatic spotting system. Using this approach we analyzed mammalian sera (rat and mouse) as biological samples to demonstrate performance. The labeling was carried out using a binary set of 2-nitrobenzenesulfonyl chloride (heavy and light), which modified tryptophan residues in sample proteins. Approximately 1600 doublet peaks were detected in the mass spectrum, some of which had more than threefold differences in their intensities. Systematic separation/spotting followed by mass analysis of the NBS-labeled peptides derived from biological samples is described for the first time. This method has proved to be an effective application of NBS-labeled peptides and can be a powerful technique for quantitative analysis of proteins expressed in biological systems.

KEY WORDS: 2-nitrobenzenesulfonyl chloride, NBSCl, automatic spotter, MALDI-TOFMS, HPLC, serum.

In proteome studies, efficient separation of proteins and/or peptides is generally indispensable for their characterization and differential analysis because of the enormous complexity of the biologically derived samples. Most proteome analyses have employed protein separation using high-resolution two-dimensional polyacrylamide gel electrophoresis (2-DE)¹ in combination with mass spectrometry. However, from the viewpoint of experimental efficiency, protein separation by 2-DE is laborious and difficult to automate. It is also difficult to separate two extreme classes of proteins, such as very large and small proteins, or very acidic and basic proteins. To overcome these problems, protein analysis using isotope-coded reagents together with liquid chromatographic (LC) separation has attracted increasing attention in recent years. The combination of chemical labeling,

efficient separation/automatic sampling, and MALDI-MS can greatly facilitate the process, and thus one can achieve a rapid and high-throughput quantitative protein analysis. To realize comparative analysis, separation still has a pivotal role even if the complexity of the peptide mixture of interest can be greatly diminished by using reagents incorporating stable isotopes. Such approaches are frequently labor-intensive and time-consuming, particularly in MALDI-MS. Automation of separation and spotting on a MALDI target plate significantly improves high-throughput analysis. In our differential analysis, we label tryptophan residues with 2-nitrobenzenesulfonyl (NBS) chloride^{2,3} (¹²C₆ as light reagent and ¹³C₆ as heavy counterpart)⁴ in combination with a reversed-phase high-performance liquid chromatography (HPLC), an automatic spotter, and a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer.

Several methods for labeling particular amino acid residues, such as an *in vivo* labeling in a ¹⁵N isotope-rich medium and an *in vitro* labeling with isotope-coded affinity tag (ICAT reagent), have been reported.^{7–10} In this paper, labeling was done with 2-nitrobenzenesulfonyl chloride (NBSCl), as described previously,⁴ which is categorized

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO: O. Nishimura, Life Science Laboratory, Analytical & Measuring Instruments Division, Shimadzu Corporation, 1, Nishinokyo-Kuwabaracho, Nakagyo-ku, Kyoto 604-8511, Japan (phone: 81-75-823-1617; fax: 81-75-823-1368; email: n_osamu@shimadzu.co.jp).

*These authors contributed equally to the project.

in an *in vitro* labeling reagent. The ICAT method modifies cysteine residue(s) in proteins, while the NBS method instead labels tryptophan residue(s). Tryptophan plays an important role in a number of biological events and is known to be one of the least abundant and most hydrophobic amino acid residues.^{11,12} The labeled tryptophan-containing peptides are more hydrophobic, and therefore separation using conventional chromatographic systems is generally efficient. Isotopic replacement of phenyl carbons makes quantitation easy because two signals are juxtaposed and devoid of isotopic overlapping, and light/heavy-labeled peptides co-elute chromatographically.

As a biological sample, serum was chosen for the present study because the use of serum for the diagnosis of a disease has been pursued and sufficient amounts of serum samples are readily available. The NBS-labeling method is advantageous, in particular, for serum analysis. In a serum sample of mouse, rat, or human, more than 50% of all protein is albumin, which has only one tryptophan residue but has as many as 35 or 36 cysteine residues. Therefore, significant reduction of spectral complexity, which leads to less redundancy of signals, is accomplished, even without albumin depletion.^{13–16}

Using the NBS method and HPLC-MALDI mass spectrometry, we analyzed serum proteins of hypertensive rat, hyperglycemic rat, and hyperglycemic mouse. In general, genetic diseases, such as hypertension and hyperglycemia, should arise from complex molecular mechanisms that involve fluctuation of expression of proteins—an abnormal level (up or down) of proteins. Since it is very difficult to pin down the cause of the diseases solely by genome analysis, dynamics of such proteins as core and immediate players in living bodies should directly be monitored.

We compared the expression levels of proteins between diseased samples and healthy counterparts, and detected pairwise peaks with a significant difference in their signal intensities between light and heavy modified peptide peaks. This is the first report that shows efficacy of a separation/automatic dispensing system when NBS-labeled peptides are used for differential analysis of expressed proteins. This study clearly demonstrated the efficacy of the separation/dispensing, which facilitated quantitative analysis by the NBS method.

MATERIALS

Rabbit glyceraldehyde-3-phosphate dehydrogenase (G3P), chicken ovalbumin, bovine α -lactalbumin, chicken egg-white lysozyme, bradykinin fragment (bradykinin 1-7), and human adrenocorticotrophic hormone fragment (ACTH 18-39) were purchased from Sigma (St. Louis,

MO). Normal rat serum (WKY/NCrj) and hypertension serum (SHR/NCrj) from 10-wk-old males, normal rat serum (Crj/Wistar), and hyperglycemic serum (GK/Crj) from 5-wk-old males were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Normal mouse serum (BKS.Cg-+Leprdb/+Leprdb/Jcl) and hyperglycemic serum (KK-Ay/Ta Jcl) from 10-wk-old males were purchased from CLEA Japan, Inc. (Tokyo, Japan). The sulfonyl halide reagent, 2-nitrobenzenesulfonyl chloride (NBSCl-¹²C₆: light form), was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and its heavy counterpart was synthesized for use. Sephadex LH-20 and were purchased from GE Healthcare Bio-Science (Piscataway, NJ). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI).

α -Cyano-4-hydroxycinnamic acid (CHCA), 3-hydroxy-4-nitrobenzoic acid (HNBA), guanidine hydrochloride, iodoacetamide, trifluoroacetic acid (TFA), tris (2-carboxyethyl) phosphine (TCEP) hydrochloride, and tris (hydroxymethyl) aminomethane (Tris) were commercially available. Water used in all experiments was purified using a MilliQ Water Purification System (Millipore Corporation, Bedford, MA).

General Procedure for Preparing NBS-Labeled Peptides¹⁷

First, two protein samples to be compared were prepared. Each sample (protein content = 100 μ g) was denatured independently with 25 μ L of 6 M guanidine-HCl and 5 mM EDTA, and was then modified with 2-nitrobenzenesulfonyl chloride (heavy for one, light for the other), resulting in proteins tagged with light or heavy NBS, respectively. The two resulting tagged protein mixtures were then combined, and the excess reagent was removed by eluting with 30% acetonitrile aqueous solution through a Sephadex LH-20 column, and dried using a vacuum concentrator. The residue was dissolved in 48 μ L of 50 mM Tris buffer (pH 8.8) containing 8 M urea. We then added 1 μ L of 200 mM TCEP and mixed gently for 30 min at 37°C; then we added 1 μ L of 500 mM iodoacetamide and mixed gently for 45 min at ambient temperature in the dark. NBS-tagged proteins were digested by adding 10 μ g of trypsin in 500 μ L of 50 mM Tris-HCl (pH 7.8) containing 5 mM CaCl₂ at 37°C for 16 h. The target fragments with tagged tryptophan in the digest were eluted through phenyl sepharose in a stepwise manner, with 1 mL of 0.1% TFA containing 10, 15, 20, 25, 30, 35, and 40% acetonitrile solutions, sequentially. The fractions eluted with 10 to 15% acetonitrile solutions were pooled and dried in a vacuum concentrator, and then were suspended in 30 μ L of 0.1% TFA solution.

Modification on Tryptophan Residue with NBSICI

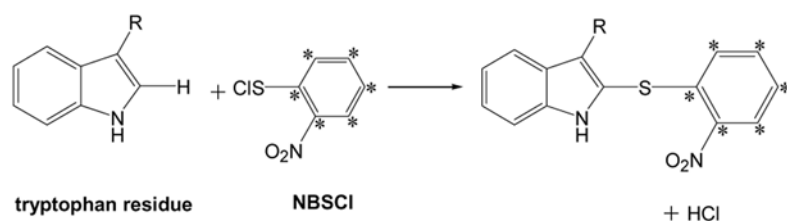


FIGURE 1

Structure and reaction of the NBS reagent. The reagent exists in two forms, heavy (contains six ^{13}C) and light (contains no ^{13}C).

NBS (2-nitrobenzenesulfonyl) method

* : six ^{13}C (heavy) or six ^{12}C (light)

The fractions eluted with 20 and 25% acetonitrile solutions and the fractions eluted with 30 to 40% acetonitrile solutions were treated in a same manner. For each of the combined fractions, 10 μL (one-third of the total amount) was provided for the LC treatment.

Sample Separation by LC System

A portion of the sample obtained by the NBS method described above was manually injected into Micro HPLC System for Proteome Analysis (Shimadzu, Kyoto, Japan). Using a Peptide CapTrap Cartridge (Michrom Bioresources, Inc., Auburn, CA), the sample was pre-concentrated and desalted with the trap column for 3 min with 0.1% TFA. Its chemical components were separated using a 0.32 mm ID \times 100 mm BetaBasic 18 Capillary Column (ThermoFinnigan, San Jose, CA). Reversed-phase liquid chromatographic (RP-LC) separation was performed with mobile phase composed of solvent A (0.1% v/v TFA and 5% v/v acetonitrile) and solvent B (0.1% v/v TFA and 90% v/v acetonitrile). LC-10ADvp μHPLC Pumps (Shimadzu, Kyoto, Japan) were used to deliver the mobile phase at the flow rate of 10 $\mu\text{L}/\text{min}$ in such a way that the B:A ratio was increased from (0.14:0.86) to (0.80:0.20) time-dependently and linearly for 30 min. The gradient composition profile was controlled by LCMS Solution SP6 Software Version 1.03 (Shimadzu, Kyoto, Japan). The UV response was monitored at 220 nm and 360 nm, where the NBS label absorbs. The portion eluted with a flow rate of 10 $\mu\text{L}/\text{min}$ was then delivered to an online AccuSpot LC-MALDI spotting robot system (Shimadzu, Kyoto, Japan), where with a flow rate of 2 $\mu\text{L}/\text{min}$, a binary mixture of matrices (8 mg/mL CHCA and 8 mg/mL HNBA in 80% aqueous acetonitrile solution/0.1% TFA (1:1))18, was mixed with the main flow from the LC system, and 12 spots of 1 μL each were deposited per minute onto

the MALDI target plate automatically. The sample was completely dried and subjected to the following mass-spectrometric measurements.

Mass Spectrometric Analysis

All of the MALDI-TOF mass spectra were obtained using an AXIMA-CFR Plus spectrometer (Shimadzu/Kratos, Manchester, UK) equipped with a 337-nm nitrogen laser and a 1.2-m drift tube. The instrument incorporates a microchannel plate-type ion detector. The acceleration potential was set to 20 kV using a gridless-type electrode. Automatic analysis was done in a positive-ion-detecting reflectron mode, where an external calibration was made with a mixture of bradykinin (1-7) and ACTH (18-39). Data acquisition and processing were controlled by Launchpad Software Version 2.4.0 (Shimadzu/Kratos, Manchester, UK). In the observed mass spectra, peak areas versus mass/electric charge (m/z) of mono-isotopic ions were calculated with Mascot Distiller Software Version 1.1.2.0 (Matrix Science, London, UK). Out of those peaks, pair peaks with 6-Da or 12-Da difference were extracted and analyzed using Conv4 NBS Software Version 1.0.0 (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

NBS Reagents and NBS Procedure

The NBS method is based on the selective introduction of a 2-nitrobenzenesulfonyl group onto the indole ring of a tryptophan side-chain (Figure 1). The overall process is shown in Figure 2. The details of the reaction are discussed in references 2–4, and the procedure is described above. We employed the improved protocol reported by Matsuo et al.¹⁷ The differences from the original procedure are mainly in denaturing of protein and enrichment of labeled peptides. For denaturing, SDS was replaced by urea, and for the enrichment process, phenyl sepharose

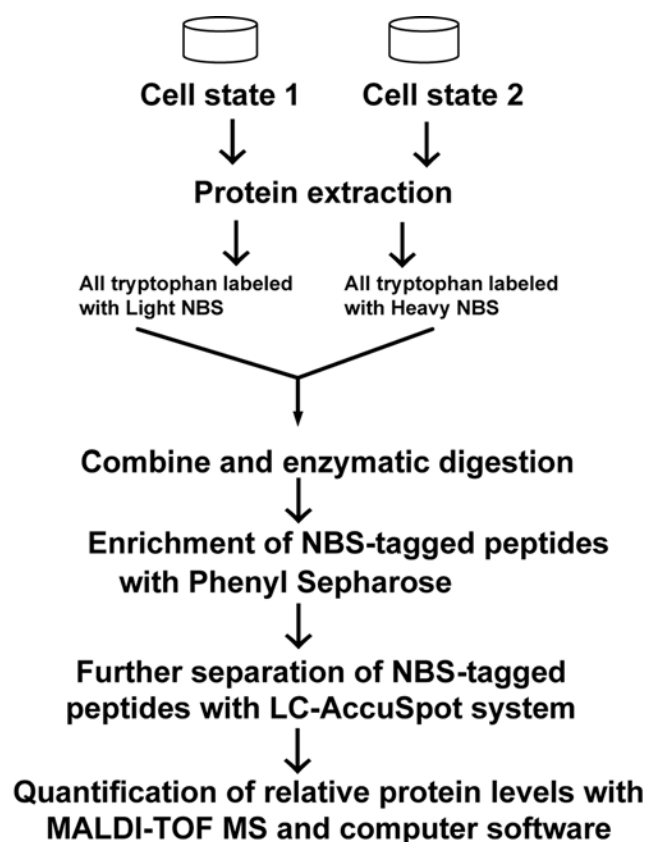


FIGURE 2

The NBS strategy in combination with HPLC-MALDI-MS system for quantifying differential protein expression. Protein samples from two conditions or states are labeled with the light and the heavy NBS reagents, individually. The samples are combined, digested, concentrated, separated with the LC-AccuSpot system, and then analyzed by mass spectrometry.

was used in place of LH-20. These changes greatly increased the recovery of NBS-tagged proteins and the efficiency in enrichment.

Efficient Separation and Recovery of NBS-Tagged Peptides by LC-MALDI System

First, we used egg-white lysozyme as a sample protein. The samples (100 μg each) were processed through the protocol described above, except for the use of the phenyl sepharose column. The enrichment was performed using a Sephadex LH-20 column (500 mg dry weight, wet volume 2 mL). The elution was performed with 30% aqueous acetonitrile, and the enriched fraction was dried using a vacuum concentrator. The resultant dried material was redissolved with 50 μL of 0.1% TFA aqueous solution; then 1 μL of the solution was injected into the Micro HPLC System as described above. In this experiment a trap column was not used. Figure 3 shows the HPLC chromatogram. Four peaks (A–D) detected at

$\lambda = 360$ nm in the range of 18–26 min were determined as NBS-labeled tryptophan containing fragments (A: GTD-VQAWIR, B: GYSLGNWVCAAK, C: WWCNDGR, D: IVSDGNGMNAWVAWR), and their H/L ratios were 1.01, 1.06, 0.95, and 0.85, respectively (Figure 4). These ratios were in good agreement with the expected ratio of 1:1. The methionine residue in the peptide (peak D) was considered to be oxidized to the sulfoxide due to the 16-Da increase of the molecular mass.

Next, this approach was applied to two of the mixture samples, each of which contains the same amount of four common proteins: rabbit glyceraldehyde-3-phosphate dehydrogenase (G3P), egg-white lysozyme, chicken ovalbumin, and bovine α -lactalbumin (each 25 $\mu\text{g}/25$ μL). Thus, the samples contain a total of 100 μg protein. One sample's proteins were labeled with light NBS, the other's with heavy NBS. Both samples were subjected to the general analytical procedure described above. The resultant peptide mixtures, after trypsin digestion, were eluted through a column in a stepwise manner, with 10, 15, 20, 25, 30, 35, and 40% acetonitrile solutions, respectively. The fractions eluted with 10 to 15% acetonitrile solutions were pooled and dried in a vacuum concentrator, and then were suspended in 30 μL of 0.1% TFA solution. The fractions eluted with 20 and 25% acetonitrile solutions and the fractions eluted with 30 to 40% acetonitrile solutions were treated in the same manner. For each of the combined fractions, 10 μL (one-third of the total amount) was used for the LC treatment.

After separation/automatic spotting onto a MALDI plate, mass spectra were acquired, and the assignment of the observed peaks was done with the theoretical m/z values because the protease and the sequences of proteins used were known. One of the examples of MALDI-TOF mass spectra observed for those fragments is shown in Figure 5, where light and heavy NBS-tagged peptides (LDQWLCEK), which were derived from α -lactoalbumin, gave pair peaks with a 6-Da difference. Relative abundances of the two peptides estimated from their peak areas ($^{12}\text{C}_6/^{13}\text{C}_6 = 1.04$) agreed well with the expected 1.00 value, indicating that the two identical but mass-differentiated peptides were well recovered and their relative intensity accurately detected by mass spectrometry. Even though the enrichment efficiency was good initially and highly improved using , not all of the labeled peptides are always detected by MALDI-MS. This is not due to sample loss but because the peptides' dynamic range and ionization efficiency greatly differ depending on their mixture composition and structures, respectively. As for the relative intensity, peak overlapping causes less satisfactory results. Therefore, separation of each peptide is a powerful measure for the detection

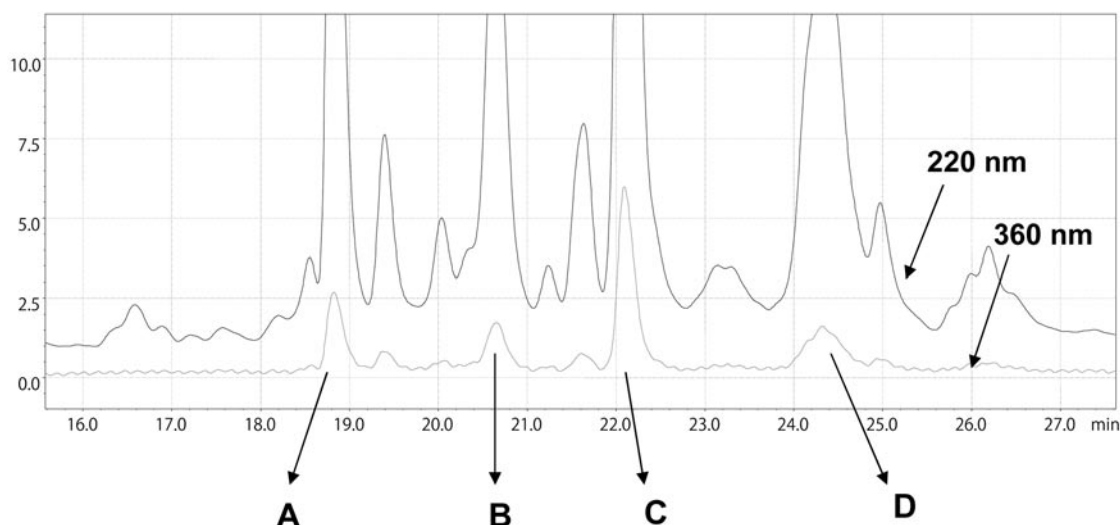


FIGURE 3

HPLC chromatogram of trypsin-digested mixture of egg-white lysozyme. Four peaks are seen in the range of 18–26 min of retention time at $\lambda = 360$ nm detection.

of minor or less ionizable peptides as well as accurate quantitation.

After LC separation, we successfully detected doublet peaks derived from peptides tagged with light and heavy NBSs in the mass spectrum. In the m/z range from 900 to 2000 we detected the peptides with high resolution. Table 1 shows that all of the theoretical, NBS-tagged fragments from the four proteins were recovered and accurately detected by LC/MALDI-TOF MS measurements. The observed ratios for the pairwise peaks were found to be in the range from 85 to 111%. The mean value of every observed ratio closely corresponded to the expected value of 100%. These experimental results demonstrated that NBS-tagged fragments were effectively enriched by a column, well separated by RP-HPLC, and detected with high sensitivity by mass spectrometry. The combination of separation and fractionation greatly facilitated the analysis of NBS-labeled peptides.

Application of the System to Quantitative Analysis of Serum Protein Expression

The analysis system worked well with the mixture of four proteins. The system was then applied to the much more complex mixture of proteins. The samples used were rat and mouse sera without any pre-treatments such as albumin depletion. The experiment was carried out as follows. First, normal as a control (WKY/NCrj) and hypertension (SHR/NCrj) serum samples were taken from rats (*Rattus norvegicus*). Each serum (total protein content = 100 μ g each, which corresponds to ca. 3 μ L of serum) was treated according to the general analytical procedure described above (light NBS labeling for the

normal and heavy NBS for the diseased). The resulting labeled peptides were subjected to the LC-MALDI-MS system and analyzed in the m/z range from 900 to 2500 Da. We detected, using the analysis software, 1787 pair peaks having 6 Da or 12 Da differences. These pair peaks arise from peptides including one or two NBS-tagged tryptophans, respectively. Out of the 1787 pair peaks, we found 14 pair peaks that differ by more than threefold in expression levels between the control and diseased samples (calculated from their peak areas). Figure 6 shows an example of the MALDI-TOF mass spectra observed for trypsin-digested peptides from the sera, where one of the pair peaks with a 6-Da difference (m/z : 1073, 1079) and an intensity ratio of 100:444 is in the enlarged panel. At the enrichment step, separation occurred concomitantly, to some extent. However, the number of detected pair peaks was not more than 100 when using serum, unless LC separation was performed (data not shown). In the rat sera of a control (Crj:Wistar) and a hyperglycemic disease with blood glucose level of 217 mg/dL (GK/Crj), we detected 1228 doublet peaks with 6- or 12-Da difference, including 7 pair peaks that showed more than threefold difference in expression based on their peak areas. Finally, using mouse *Mus musculus* sera of a control (BKS. Cg-+Leprdb/+Leprdb/Jcl) with blood glucose level of less than 300 mg/dL and hyperglycemic (KK-Ay/Ta Jcl) with more than 500 mg/dL, we detected 1670 pair peaks with 6-Da difference, including 11 pair peaks that differ by more than threefold in expression based on their peak areas. Light and heavy NBS-tagged peptide fragments from rat and mice sera are compared and summarized in Table 2.

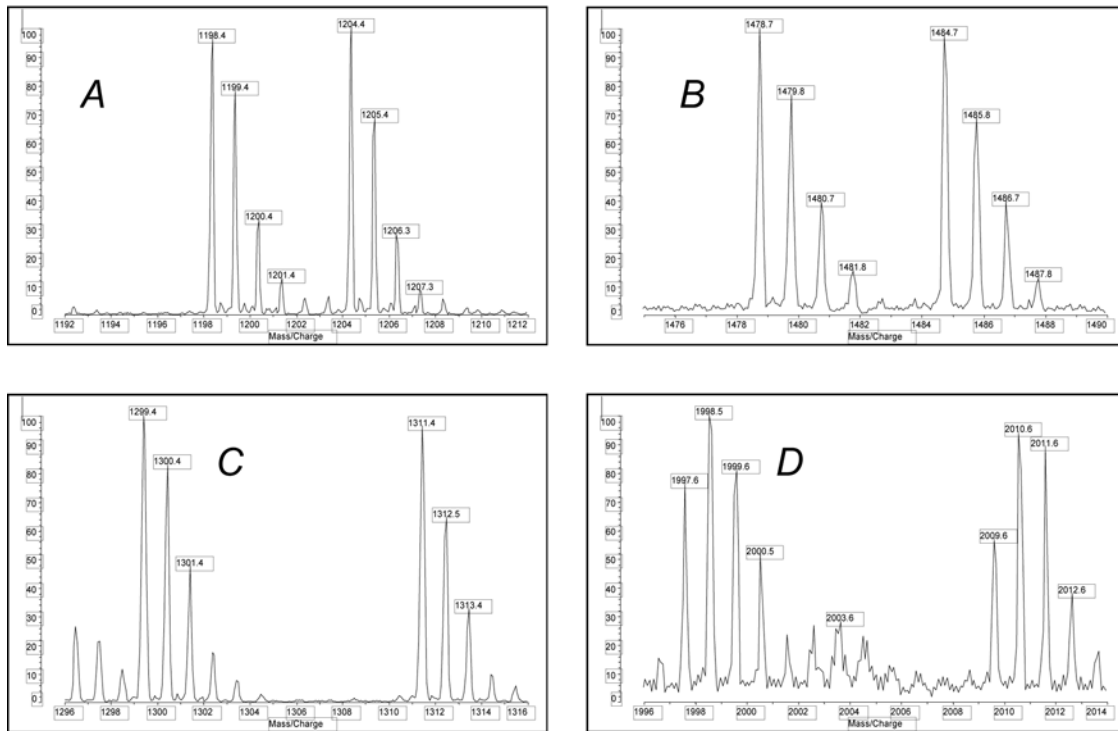


FIGURE 4

MALDI-TOF mass spectra of the four peaks in the HPLC chromatogram (Figure 3). Four theoretical digested fragments containing NBS-tagged tryptophan(s) up to m/z 2000 are determined (A: GTDVQAWIR, B: GYSLGNWV-CAAK, C: WWCNDGR, D: IVSDGNGM*NAWVAWR). *Met is oxidized.

As stated already, ionization efficiency and concentration of each peptide in a mixture greatly affect the detectability of peptides of interest, and signal overlapping reduces the accuracy of quantitation. Hence, further separation after the enrichment step should be required for the detection of minor proteins as well as for accurate quantitation. Here we showed that HPLC separation and automatic fractionation greatly improved the detectability

of such minor proteins, providing us with a powerful tool for quantitative analysis of proteins derived from biological sources.

CONCLUSIONS

An investigation into the efficacy of a separation/dispensing system followed by MALDI-TOF MS for comparison analysis of the NBS method was carried out using single

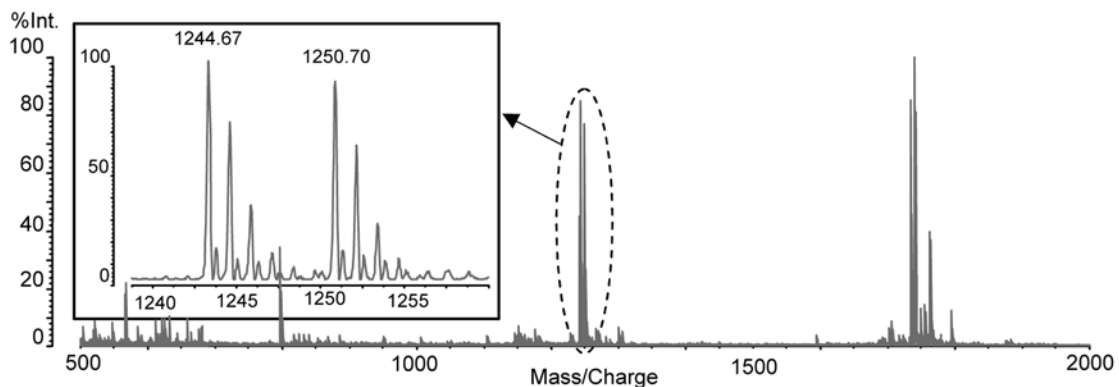


FIGURE 5

Relative quantification of trypsin-digested peptides from a four-protein mixture. One example of the MALDI-TOF mass spectra obtained for HPLC-purified/automatically spotted samples. The circled pairwise peaks correspond to the fragments; LDQWLCEK is from bovine α -lactalbumin. The inset shows an expanded view of the pair peaks.

TABLE 1

Quantification of Pair Peaks of NBS-tagged Peptides from a Four-protein Mixture

Gene Name ^a	Peptide Sequence Identified ^b	Observed Ratio (¹² C ₆ / ¹³ C ₆)	Expected Ratio	Mean±SD	% Error
LCA_BOVIN	I <u>W</u> CK	1.01	1	1.04±0.03	4
	LDQ <u>W</u> LCEK	1.04			
	VGINY <u>W</u> LAHK	1.06			
OVAL_CHICK	GL <u>W</u> EK	0.99	1	0.96±0.10	4
	LTE <u>W</u> TSSNVMEER	0.85			
	ELINS <u>W</u> VESQTNGIIR	1.04			
G3P_RABIT	L <u>W</u> R	0.96	1	0.99±0.04	1
	LIS <u>W</u> YDNEFGYSNR	1.02			
	<u>W</u> GDAGAEYVVESTGVFTTMEK	(not determined)			
LYC_CHICK	GTDVQ <u>W</u> IR	1.05	1	1.02±0.08	2
	<u>W</u> WCNDGR	1.11			
	GYSLGN <u>W</u> VCAAK	1.01			
	IVSDGNGMNA <u>W</u> VAVR	0.89			

^a Gene names given by Swiss Prot nomenclature (<http://www.expasy.ch>).

^b Each tryptophan residue in the peptides (underlined) is labeled with NBS moiety.

protein sample and quaternary mixture samples. It was shown that clear separation and efficient fractionation onto the MALDI target plate resulted in the detection of every theoretical peak and good correlation of the signal ratios between the expected and the observed peaks. Thus, successful detection of the labeled peaks was realized. This system was then applied to complex biological samples for comparison of expressed proteins. We used sera of hypertensive rat, hyperglycemic rat, and hyperglycemic mouse with no separation/purification or protein extraction

prior to use. From a proteome comparative study between the diseased- and healthy-state samples we found that a number of pair peaks with significant differences in their expressed protein levels was detected. A detailed description of the pair peaks detected is beyond the scope of this article. The current study was aimed at evaluating the technical feasibility of separation/dispensing of NBS-labeled peptides for MALDI-TOF mass spectrometry.

The present method of NBS labeling in conjunction with HPLC-automatic spotting apparatus, followed by

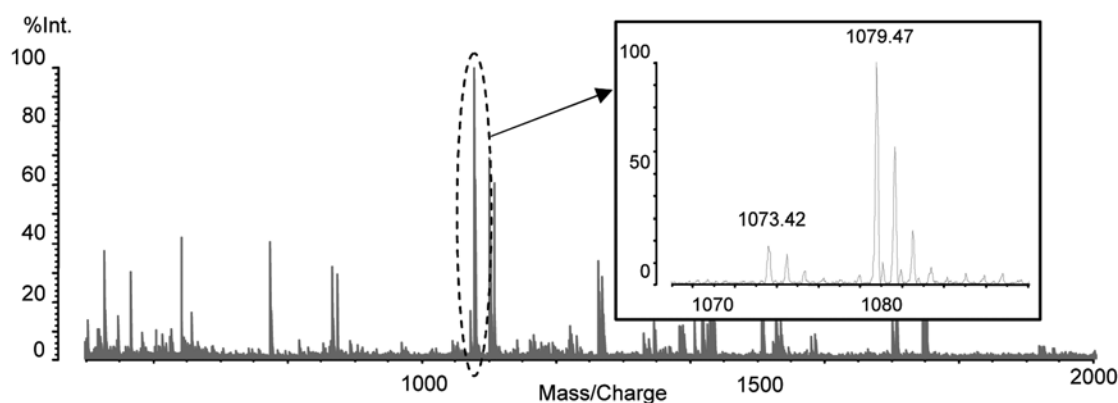


FIGURE 6

Relative quantification of trypsin-digested peptides from the normal and hypertension rat sera proteins. One example of the MALDI-TOF mass spectra obtained for HPLC-purified/automatically spotted samples. The circled pairwise peaks shows ca. 1:5 ratio for the difference in expression. The inset shows an expanded view of the pair peaks.

TABLE 2**Quantification of Pair Peaks of NBS-Tagged Peptides from the Normal and Diseased Mammalian Sera**Control Rat → NBS light reagent³⁴⁰ : Hypertension Rat → NBS heavy reagent

No	<i>m/z</i> (NBS light)	<i>m/z</i> (NBS heavy)	Ratio (NBS light : heavy)	Mass difference
1	1051.9	1057.8	100 : 490.5	6
2	1056.5	1062.5	100 : 27.5	6
3	1073.4	1079.4	100 : 443.8	6
4	1116.5	1122.5	100 : 463.9	6
5	1117.4	1123.4	100 : 549.8	6
6	1133.3	1139.3	100 : 591.3	6
7	1165.1	1171.1	100 : 518.3	6
8	1181.5	1187.5	100 : 531.5	6
9	1260.5	1266.5	100 : 430.3	6
10	1275.4	1281.4	100 : 18.4	6
11	1479.8	1485.8	100 : 28.8	6
12	1948.8	1954.8	100 : 23.4	6
13	2016.9	2022.9	100 : 17.3	6
14	2362.9	2368.9	100 : 20.9	6

Control Rat → NBS light reagent : Hyperglycemic Rat → NBS heavy reagent

No	<i>m/z</i> (NBS light)	<i>m/z</i> (NBS heavy)	Ratio (NBS light : heavy)	Mass difference
1	1030.6	1036.7	100 : 28.5	6
2	1056.9	1062.9	100 : 628.8	6
3	1523.2	1529.1	100 : 684.8	6
4	1669.1	1675.2	100 : 702.6	6
5	1691.3	1697.3	100 : 510.0	6
6	1949.1	1955.2	100 : 537.1	6
7	2016.5	2022.5	100 : 609.6	6

Control Mouse → NBS light reagent : Hyperglycemic Mouse → NBS heavy reagent

No	<i>m/z</i> (NBS light)	<i>m/z</i> (NBS heavy)	Ratio (NBS light : heavy)	Mass difference
1	1083.1	1089.1	100 : 21.6	6
2	1359.7	1365.7	100 : 314.0	6
3	1384.5	1390.5	100 : 631.5	6
4	1494.6	1500.6	100 : 305.2	6
5	1605.9	1612.0	100 : 305.2	6
6	1622.6	1628.6	100 : 332.0	6
7	1773.0	1779.0	100 : 767.8	6
8	1887.7	1893.7	100 : 312.2	6
9	2021.8	2027.1	100 : 355.3	6
10	2150.1	2156.1	100 : 397.0	6
11	2281.4	2287.5	100 : 31.8	6

mass measurements with MALDI-TOF mass spectrometer, has been proven useful for the quantitative analysis of expressed proteins.

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