Development of Iodoacetyl-Based Ionic Mass Tags for Improved Sensitivity in the Detection of Cysteine-Containing Peptides by MALDI-TOF MS

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Abstract

We developed and characterized six new ionic mass tags that bond to cysteine residues and enable high-sensitivity analysis of peptides. Each mass tag molecule was designed with the following structural characteristics: 1) an iodoacetyl group that binds to thiols; 2) hydrophilic properties that reduce losses due to physical adsorption of the sample; 3) a tertiary amine, quaternary ammonium, or guanidino group to retain strong proton affinity; and 4) no amide bonding to minimize fragmentation caused by collision-induced dissociation. We analyzed peptides for their MS sensitivity using the new mass tags and achieved improvements in sensitivity of approx. 2 to 200 times the sensitivity obtained with a carbamide-methylated peptide control sample. The new ionic mass tags are ideal for analytical mass spectrometry of hydrophobic peptides, low-abundance biomarkers, and circulating hormones.

1. Introduction

Living systems contain a wide dynamic range of proteins, and the accompanying molecular complexity contributes to maintaining the miraculous balance observed in biological phenomena. Overcoming that complexity is an extremely large challenge for proteomics, which involves the exhaustive analysis of the proteome[1-2]. With current MS technology available, it is difficult to detect proteins present at low levels amongst highly abundant proteins. Many different techniques have therefore been developed for the pretreatment of samples before introduction to the mass spectrometer, including multi-stage separations and affinity purification[3-8]. Derivatizations that target cysteine are the most common form of irreversible modification used for biochemical binding due to the universal distribution of cysteine residues in diverse proteomes, the low abundance of the cysteine amino acid, and high chemical reactivity of the thiol group9). To date, a diverse range of reagents that typically contain a maleimide group, iodoacetyl group, or vinyl group have been used to introduce a functional group with chemical affinity, as crosslinking reagents, in fluorescent probes as activity markers, and in quantitative analysis. An *in silico* analysis that we performed using the human proteins database registered in the International Protein Index (IPI) found 97 % of human proteins contain at least one cysteine residue, and that 17 % of predicted tryptic fragments also contain at least one cysteine residue. This means that predicting a proteome based only on the fraction of its peptides that contain cysteine could result in a well-described proteome. The results also suggest that samples can be reduced to about one-fifth of their original complexity without loss of proteome coverage. The selection of cysteine-containing proteins as the subject of proteomic analysis is likely to contribute to the field of proteomics through a bottom-up approach.

(Received December 17, 2012)

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2. Experiments

2.1 Reagents

All commercially available reagents were used without further purification. Bovine insulin (MW = 5734), $P_{14}R$ synthetic peptide (MW = 1533), ammonium bicarbonate, and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, US). NC4 CLAC-P peptide (collagen-like Alzheimer amyloid plaque component precursor) 641-654 (LGPDGLP-MPG CWQK, MW = 1499) and S26C amyloid-beta 17-40 peptide (LVFFAEDVGC NKGAIIGLMV GGVV, MW = 2409) were purchased from Anaspec (Fremont, CA, US). Alpha-fetoprotein was purchased from SCIPAC (London, UK). High-purity alpha-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Shimadzu GLC (Tokyo, Japan). Other solvents and reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2 Mass Tag Synthesis

For details on the six new cysteine mass tags, see the "References" section [10].

2.3 Labeling Peptides with Mass Tags

Peptides were dissolved in an aqueous solution of 50 % acetonitrile containing 0.05 % trifluoroacetic acid to a final concentration of 100 pmol/μL. An amount of this solution that contained 200 pmol of peptide was then added to 10 μL of an aqueous solution of 100 mM ammonium bicarbonate and 10 mM dithiothreitol, and the resulting mixture was heated at 56 °C for 60 minutes to reduce the disulfide bonds. After the reduction reaction ended, 15 μL of a 100 mM ammonium bicarbonate and 50 mM mass tag reaction solution was added to the mixture and stirred gently at room temperature for 45 minutes to allow s-alkylation. Trifluoroacetic acid was then added to a final concentration of 1 % to end the peptide labeling reaction.

2.4 Mass Spectrometry and Data Analysis

2 pmol of mass tag-labeled peptides was diluted in 0.1 % trifluoroacetic acid, purified using ZipTip C18 (Millipore, Billerica, MA, US) according to a standard protocol, spotted directly onto a stainless steel MALDI target, and then air-dried. CHCA was added to an aqueous solution of 50 % acetonitrile and 0.1 % trifluoroacetic acid to make a CHCA concentration of 5 mg/mL, then P14R was added to this mixture to a final concentration of 300 fmol/μL.

This matrix solution was then placed over the spotted samples on the MALDI target. MALDI-TOF MS was performed using the AXIMA Performance (Shimadzu Corporation, Kyoto, Japan). Data was acquired in linear positive ion mode using a UV nitrogen laser (337 nm) and in automatic measurement mode at an accelerating voltage of 20 kV and with 300 profiles/run. The intensity of target peptide peaks was normalized using the peak intensity of $P_{14}R$, which was added as an internal standard. Outliers were discarded by determining the 35 % trimmed mean of the normalized peak intensity (n = 12). The resulting spectra were then evaluated statistically.

2.5 Serum Peptidome Analysis

15 µL of blood serum from a seven-year-old C57BL/6N mouse (Charles River Laboratories, Yokohama, Japan) was denatured in 70 % acetic acid at 100 °C for 30 minutes. After centrifugation (20,000 g for 60 minutes), the supernatant was filtered through a 0.45 µm Ultrafree-MC (Millipore), and the resulting sample was separated by gel filtration chromatography on a Superdex Peptide PC 10/300 GL (GE Healthcare, Waukesha, WI, US). Eluent fractions were analyzed using an aqueous solution of 50 mM ammonium acetate (pH 7) and 20 % acetonitrile at a flowrate of 0.35 mL/min. The peptide fraction below 6 kDa was collected using the external standards ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) and concentrated using a centrifugal evaporator. 5 pmol of each NC4 CLAC-P peptide (carbamide-methylated or mass tag (e)-labeled) was added to the prepared serum peptidome. Each sample was purified using MonoTip C18 (GL Sciences, Tokyo, Japan). The serum peptidome was separated and analyzed using the Prominence nanoLC system (Shimadzu), MU701 capillary UV-VIS detector (GL Sciences), and AccuSpot MALDI spotter (Shimadzu). The following HPLC conditions were used:
Eluent A: 0.5 % acetonitrile containing 0.1 % heptafluorobutyric acid
Eluent B: 80 % acetonitrile containing 0.1 % heptafluorobutyric acid
Gradient program: 1 % B (35 min), 1-12 % B gradient (10 min), 12-52 % B gradient (135 min), and 52-100 % B gradient
Column: Presto FF-C18 (non-porous 2 µm silica, 0.5 × 150 mm, Imtakt Corporation, Kyoto, Japan)
Flowrate: 1.6 µL/min
Interval between MALDI target spots: 20 sec/well
MALDI-TOF MS was performed using the AXIMA Performance. Data was acquired in linear positive ion mode and in automatic measurement mode with 600 profiles/run. All MS spectra were normalized using the peak intensity of the P14R internal standard.

3. Results and Discussion

To date, during our research into the development of functional mass tags, we have used mass tags to improve MS sensitivity, efficiently analyze variability in gene expression, and analyze the amino acid sequence of proteins[11-13]. This report describes newly developed cysteine mass tags that can be used as sensitivity enhancers. The molecular design of these cysteine mass tags has four features, as illustrated in Fig. 1: (1) an iodoacetyl structure for binding to thiol groups with high sensitivity and minimizing side reactions with other amino acid residues; (2) a hydrophilic structure to avoid peptide recovery losses caused by physical adsorption; (3) tertiary amine, quaternary ammonium, or guanidine groups to confer strong proton affinity. (There are already a number of reports on the technique of increasing the detection efficiency of MS by introducing a fixed positive charge to the molecule[14-16].); and (4) an ester bond between the iodoacetyl group and positive ion receptor to minimize mass tag fragmentation due to collision-induced dissociation17). Six cysteine mass tags were synthesized incorporating these four features in their molecular structures (Fig. 2).
Four model peptides (insulin alpha-chain and beta-chain, NC4 CLAC-P, and S26C beta-amyloid 17-40) were used to optimize the labeling reaction conditions of the synthesized mass tags and verify their enhancement of the MS signal. The optimal reaction conditions were 45 minutes at room temperature in the dark, during which almost all reactions with cysteine were completed. MS signals were undetectable for unreacted peptides and side products derivatized to non-cysteine amino acid residues (Fig. 3).
The sample area of a MALDI target is well known to have "sweet spots" that generate extremely strong ion signals and "non-sweet spots" that generate almost no signal—a phenomenon that is reported to be affected by the state of co-crystallization between the analyte and the matrix[18]. Consequently, it must always be bore in mind that abnormal values will occur when MALDI-TOF MS is used for quantitative analysis. Ionization efficiency also varies substantially with different peptide sequences. We therefore devised a new method for quantitating peptides using MALDI-TOF MS that is composed of three steps: (1) obtaining multipoint data (n = 12) by normalizing individual sets of data with an internal standard; (2) discarding outlier peak intensities by using a 35 % trimmed mean; and (3) statistical analysis to evaluate the significance of sensitivity improvements. This analytical technique makes highly accurate quantitative analysis possible using MALDI-TOF MS. Typical MS spectra are shown in Fig. 4, where $P_{14}R$ is used as the internal standard.

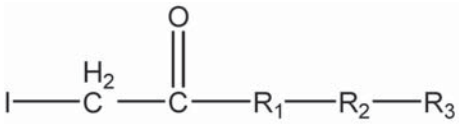

fig 1. Mass tag structure with multi-functional property
R_1; NH (amide), O (ester)
R_2; hydrocarbon (linker), ethyleneglycol (hydrophilic linker)
R_3; tertiary amine, quaternary amine, guanidine, triazine (proton acceptor)

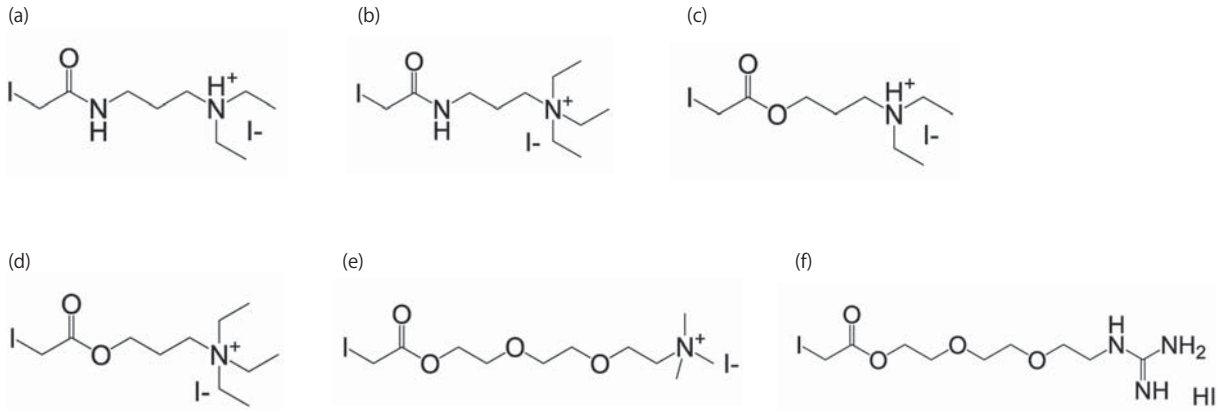

Fig 2. Structure of cysteine mass tags with CLogP value of derivatized form
(a) DE-IAA, 1-(N,N-diethylamino)-3-iodoacetamidopropane hydroiodide (0.849)
(b) TE-IAA, 3-iodoacetamidopropyltriethylammonium iodide (-2.890)
(c) DE-IA, 1-(N,N-diethylamino)-3-iodoacetoxypropane hydroiodide (1.857)
(d) TE-IA, 3-iodoacetoxypropyltriethylammonium iodide (-1.882)
(e) TM-DEG-IA, 8-iodoacetoxy-3,6-dioxaoctyltrimethylammonium iodide (-3.538)
(f) Gu-DEG-IA, 8-guanidino-1-iodoacetoxy-3,6-dioxaoctane hydroiodide (-1.091)

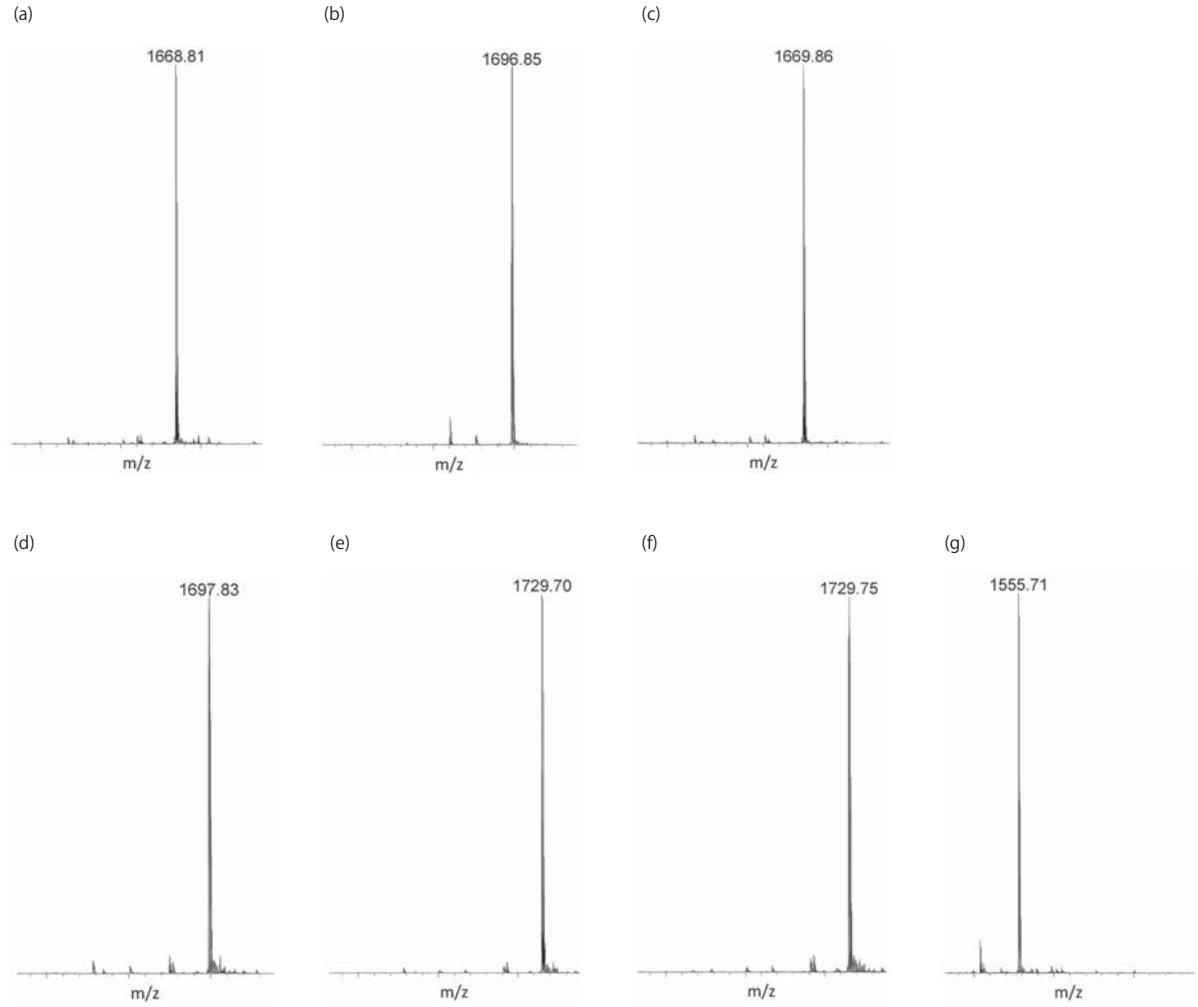

Fig 3. MS spectra of tag-derivatized NC4 CLAC-P peptide
The tags used were : (a) DE-IAA : m/z 1668.81 as [M+H]+ , (b) TE-IAA : 1696.85 as [M]+, (c) DE-IA : 1669.86 as [M+H]+, (d) TE-IA : 1697.83 as [M]+, (e) TM-DEG-IA : 1729.70 as [M]+, (f) Gu-DEG-IA : 1729.75 as [M+H]+, and (g) IAA-derivatized NC4 CLAC-P control: 1555.71 as [M+H]+

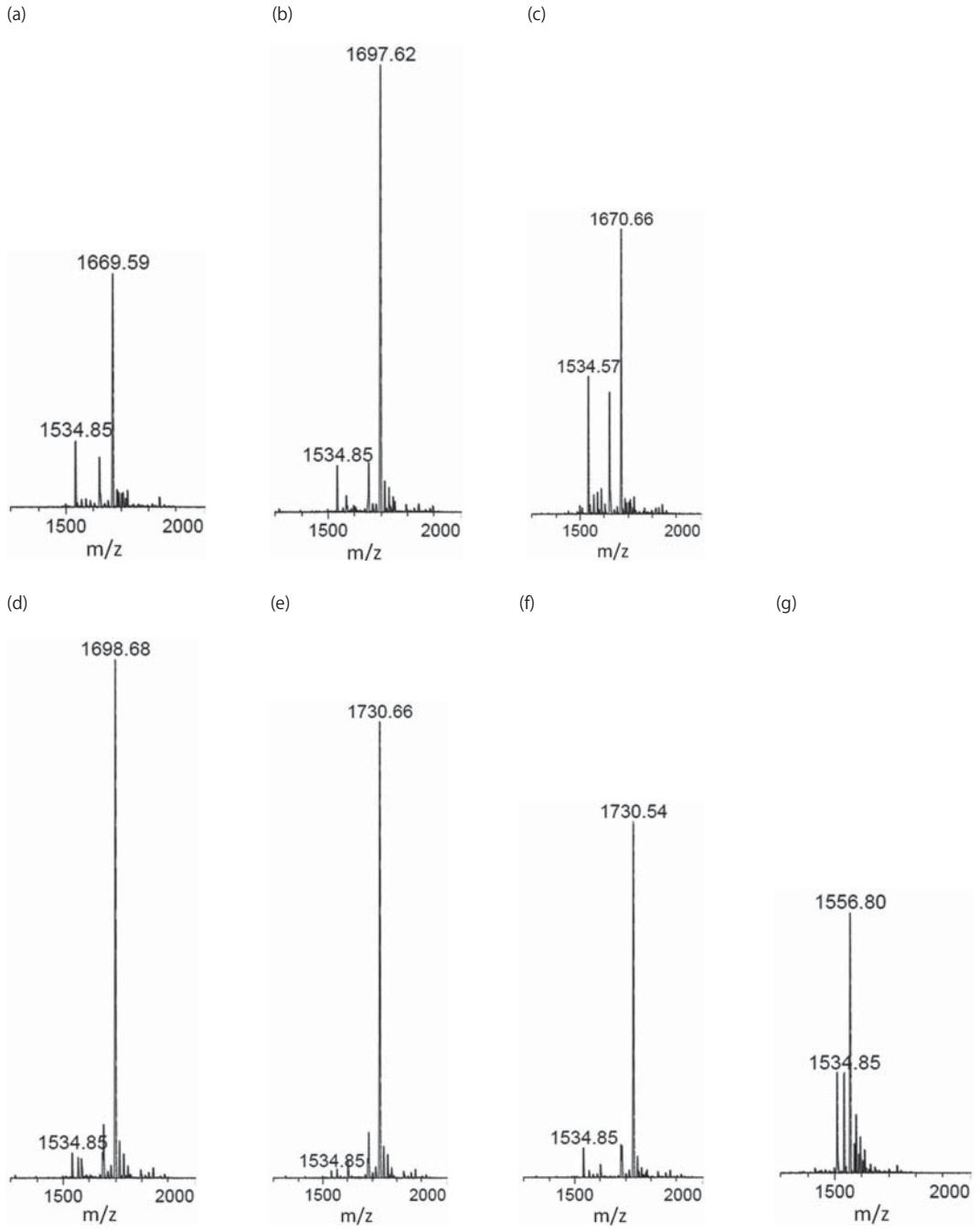

fig.4 MS spectra of tag-derivatized NC4 CLAC-P peptide along with $P_{14}R$ as an internal standard for quantitative peptide analysis

The tags used were : (a) DE-IAA: m/z 1669.59, (b) TE-IAA: 1697.62, (c) DE-IA: 1670.66, (d) TE-IA: 1698.68, (e) TM-DEG-IA: 1730.66, (f) Gu-DEG-IA: 1730.54, and (g) IAA-derivatized NC4 CLAC-P as a control: 1556.80. Mass numbers are shown as averaged in this figure.

In terms of improving the sensitivity of MS, the quaternary ammonium tags (b and d) were more effective than the tertiary amine tags (a and c). Comparing the quaternary ammonium tag (e) and guanidine tag (f) shows the quaternary ammonium tag was more effective. Verifying these effects using peptides of various amino acid sequences revealed that the quaternary ammonium tag (e) improves sensitivity most remarkably among the six mass tags. Table 1 summarizes the results of the improvements. By using the mass tags developed in this research for MS analysis of peptides, we successfully improved sensitivity by a statistically significant 2 to 200 times over the carbamide-methylated peptide control sample.

Next, we evaluated the sensitivity improvements from using the mass tags with fragments from in-gel tryptic digestion of alpha-fetoprotein (AFP). AFP is a known protein biomarker of liver cancer[19]. Particular attention has been focused on the lectin 3-fraction of AFP (AFP-L3 fraction) as a specific biomarker that distinguishes between liver cancer and cirrhosis. Table 2 reveals that, even given the fact that the tryptic digest is a peptide fragment with high ionization efficiency, the sensitivity improvements depend significantly on the amino acid sequence of the peptide. This is probably because the rate of recovery of the peptide fragment from the gel also depends significantly on its physicochemical properties, which may substantially affect the absolute amounts to be used during analysis. The hydrophilic regions of the synthesized mass tags probably also help improve the rate of recovery of the peptide from the gel. We also discovered peptide fragments only detectable when labeled with the new mass tags. In addition, some data, not shown here, indicated that the new mass tags are also useful when used with peptides having an amino acid sequence that is difficult to detect with MS due to an absence of a basic amino acid at the c-terminal, contributing to a dramatic improvement in detention efficiency. The new mass tags show great potential for the development of new biomarkers through mass spectrometry.

Table 1 Enhanced sensitivity ratio of mass tag-derivatized peptides

Ratios (enhanced ratio) of tag-derivatized peptides are shown compared to carbamydomethylation (CAM) control with respective p-values.
Insulin alpha and beta, derived from reductive alkylation of insulin
NC4, NC4 CLAC-P 641-654 (collagen-like Alzheimer amyloid plaque component precursor)
S26C, S26C beta-amyloid 17-40
Tag IDs are described in Figure 2.

		Mass Tag ID					
Peptide		(a)	(b)	(c)	(d)	(e)	(f)
Insulin alpha	Ratio	44.2	58.0	18.5	121.1	222.5	167.7
	p-value	1.40E-09	1.13E-12	3.59E-11	5.65E-12	3.22E-06	6.02E-10
Insulin beta	Ratio	6.4	28.2	4.4	58.1	58.3	30.8
	p-value	2.92E-09	2.38E-12	6.47E-16	2.54E-12	1.34E-14	4.22E-10
NC4	Ratio	1.2	3.0	1.3	25.8	33.5	12.4
	p-value	9.89E-03	3.42E-09	4.51E-03	1.08E-08	1.57E-08	2.17E-07
S26C	Ratio	1.4	24.2	1.4	16.6	16.5	11.1
	p-value	0.027	1.49E-05	0.010	5.08E-06	7.54E-06	1.94E-05

Table2 Result of creating calibration curve of VP standard samples calculated from derivative spectrum chromatogram
Each peptide intensity was normalized by highest peak of m/z 15829 signal (aa. 348-360 NIFLASFVHE YSR) in the same MS spectra.
Cys; Number of cysteine in each peptide sequence
ND*; Not detected
P**; Detection of probe-coupled peptides, but not detected from control

Position	Cys-peptide sequence of AFP	#Cys	(a)	(b)	(c)	(d)	(e)	(f)
5-42	NEYGIASILD SYQCTAEISL ADLATIFFAQ FVQEATYK	1	ND*	ND*	ND*	ND*	ND*	ND*
50-83	DALTAIEKPT GDEQSSGCLE NQLPAFLEEL CHEK	2	0.5	0.2	ND*	ND*	1.7	ND*
89-102	YGHSDCCSQS EEGR	2	1.4	0.2	ND*	0.0	0.0	ND*
103-110	HNCFLAHK	1	2.5	8.9	0.0	1.5	2.5	0.0
111-136	KPTPASIPLF QVPEPVTSCE AYEEDR	1	0.6	0.0	0.2	0.0	0.01	ND*
169-175	IIPSCCK	2	14.6	11.0	0.6	0.0	0.7	ND*
176-186	AENAVECFQT K	1	3.3	20.7	0.0	38.7	29.8	3.1
196-209	ESSLLNQHAC AVMK	1	26.7	97.5	5.3	103.4	44.5	0.8
239-252	LVLDVAHVHE HCCR	2	1.1	0.1	0.0	0.0	0.1	ND*
253-264	GDVLDCLQDG EK	1	4.3	13.9	ND*	31.4	24,8	4.6
265-279	IMSYICSQQD TLSNK	1	P**	P**	P**	P**	P**	P**
280-285	ITECCK	2	916.6	962.9	0.8	6.7	538.5	192.3
292-313	GQCIIHAEND EKPEGLSPNL NR	1	0.7	0.05	ND*	0.5	0.8	0.2
365-377	CFQTENPLEC QDK	2	243.7	23.0	44.3	7.9	10.4	31.9
396-402	SCGLFQK	1	2.5	13.8	0.1	3.7	2.3	0.1
435-449	MAATAATCCQ LSEDK	2	P**	P**	P**	P**	P**	P**
450-468	LLACGEGAAD IIIGHLCIR	2	1.0	0.2	0.0	0.0	0.1	0.0
469-489	HEMTPVNPGV GQCCTSSYAN R	2	0.3	0.0	ND*	0.0	0.0	0.0
490-511	RPCFSSLVVD ETYVPPAFSD DK	1	0.8	0.2	0.3	ND*	ND*	ND*
517-530	DLCQAQGVAL QTMK	1	2.6	15.1	ND*	18.4	18.8	1.4
563-579	CCQGQEQEVC FAEEGQK	3	1.5	0.7	ND*	0.0	0.1	0.2

We also analyzed serum peptidome to verify how much sensitivity is improved by using the mass tags for proteomics. Diagnostic techniques employing multiple biomarkers in blood serum and blood plasma hold great potential as clinical applications of mass spectrometry, and of those techniques, peptidome analysis is being increasingly recognized as most effective in searching for biomarkers[20]. The results of the peptidome analysis are shown in Fig. 5. The improvements in sensitivity were verified by comprehensively analyzing about 700 serum peptides, using an internal standard to obtain definite quantitative results, and adding a carbamide-methylated peptide control sample and peptides labeled with mass tag (e). The results demonstrated that sensitivity is improved by labeling peptides with the mass tags even in an extremely complex sample such as serum peptidome.

The mass tag (e) likely achieves high sensitivity in the mass spectrometric analysis of peptides by improving peptide recovery rates due to its hydrophilic linker and by improving sensitivity due to the attachment of positive ions to peptide molecules. The peptides NC4 and S26C play a role in Alzheimer's disease, which is caused partly by tissue fibrosis and plaque formation[21]. Tissue fibrosis, in particular, is an important histopathological indication associated with progression of the disease[22]. The new mass tags can be used in the high-sensitivity analysis of clinically valuable molecules with a hydrophobic domain susceptible to fibrosis. Also, peptides of biological or clinical significance are often hormones that circulate in the bloodstream, such as insulin, natriuretic hormone, and growth factors, or peptide fragments that have undergone disease-specific cleavage[23]. These peptides are present in very small quantities and often contain a strongly hydrophobic surface as a result of their unique steric structure. Some of them also have no c-terminal basic amino acid, which makes a high-sensitivity analysis difficult to perform. The further development of methods that improve the sensitivity of peptide analysis with mass spectrometry will lead to the development of valuable biomarkers and the discovery of unknown biological systems. We consider cysteine-containing peptides, in particular, to be important candidates for detection, and anticipate that they will find application in quantitative analysis by means of stable isotope labeling or specific-fragment indices[24].

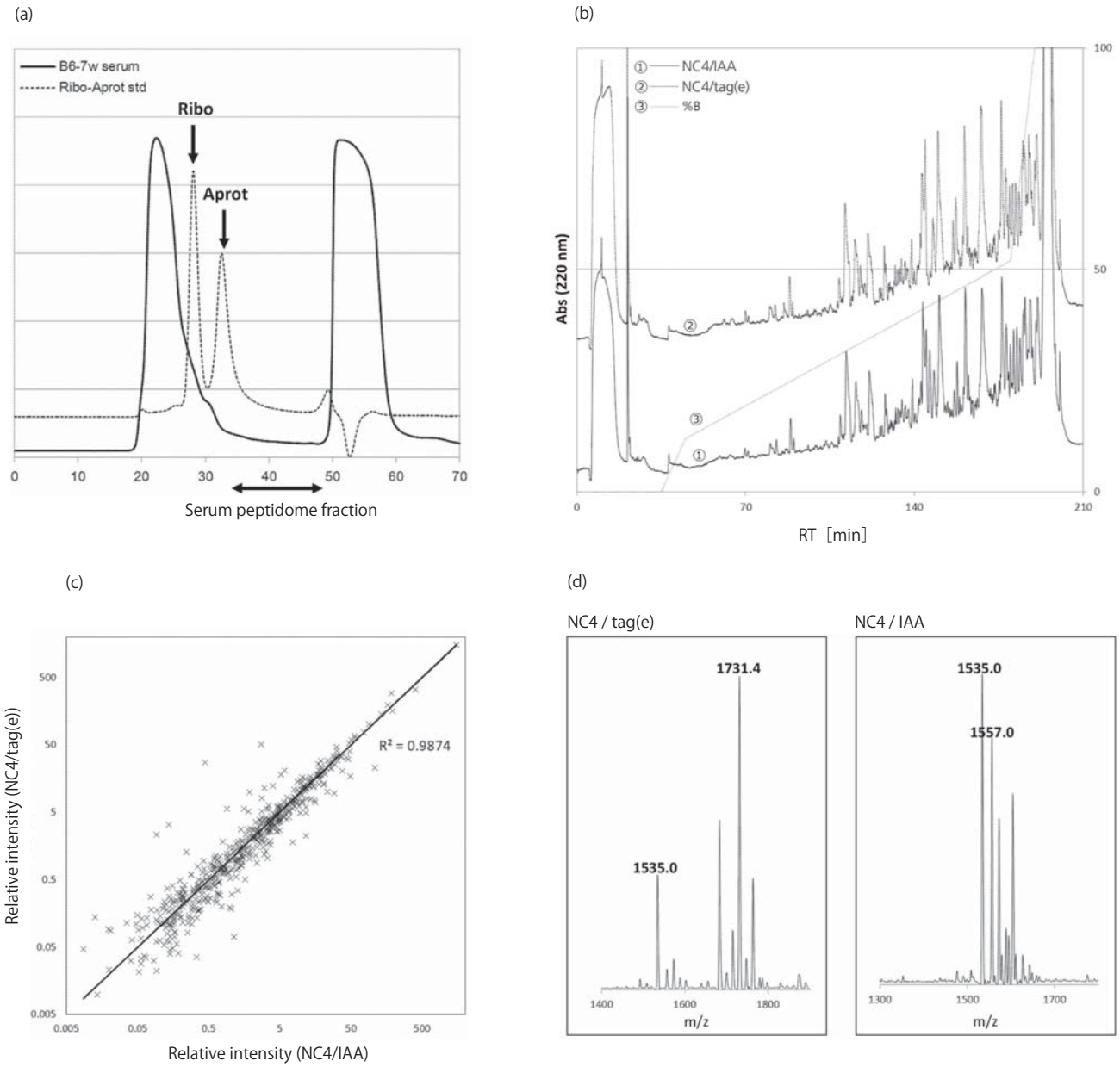

Fig 5. Enhanced sensitivity by tag-derivatized peptide in serum peptidome

(a) UV (220 nm)-absorption spectra of serum protein by size exclusion chromatography (SEC). Solid line shows the profile of C57B6-7w mice serum, and dotted is the molecular weight standard. The peak of ribonuclease A (Ribo) and aprotinin (Aprot) are indicated by arrows. Serum peptidome fraction is shown as side arrows.

(b) UV (220 nm)-absorption spectra of serum peptide fraction by reverse-phase chromatography. Left axis shows the UV-absorption and right is the percentage of solvent B concentration. Solid and dotted lines are the profile of serum peptidome spiked with NC4/IAA peptide and NC4/tag (e) peptide, respectively. The spotting point on MALDI target is from 65 min to 195 min for 20 sec/well interval.

(c) Scatter plot of peptide intensity from serum peptidome analysis. Horizontal and vertical axes indicate peptide intensity from peptidome spiked with NC4/IAA peptide and NC4/Tag-e peptide, respectively.

(d) Representative MS spectra of IAA-derivatized NC4 peptide and tag (e)-derivatized NC4. Enhanced sensitivity ratio by tag (e) derivatization is about 6.2-fold.

4. Conclusion

We carried out the molecular design and synthesis of six new cysteine-binding ionic mass tags to improve the sensitivity of peptide analysis by mass spectrometry. Of these, mass tag (e), with a quaternary ammonium and hydrophilic linker, caused the greatest increase in sensitivity. The six novel mass tags are expected to be applicable in MS analysis of hydrophobic peptides, low-abundance biomarkers, and circulating hormones.

This research is supported by the Japan Society for the Promotion of Science through its "Funding Program for World-Leading Innovative R&D on Science and Technology" (FIRST Program). Cysteine mass tag synthesis was performed with the support of Dr. Katsuya Noguchi (Dojindo Molecular Technologies).

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