

Table 1 (Continued)

Cell line	HDAC inhibitor (μM)	Apoptotic cell ^a (%)	Cell cycle distribution ^a (%)			
			sub G ₁	G ₁	S	G ₂ /M
ILKM-2	Untreated control	7.35	4.07	58.61	13.05	22.89
	SAHA					
	3 μM	7.84	8.97	61.53	6.23	22.12
	30 μM	21.41	20.63	51.10	12.71	13.88
	NCH-51					
	3 μM	8.71	6.04	63.21	6.93	22.45
	30 μM	30.04	20.54	51.45	12.15	15.13

Abbreviations: HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid.
^aEach value shows the average of 20000 cells counted.

NCH-51 induces cytotoxic effect through the modulation of intracellular ROS

From the results of proteome analyses, NCH-51 appeared to promote the expression of antioxidant molecules, either at the transcriptional or post-transcriptional level, which prompted us to examine the effect of NCH-51 on the levels of ROS accumulation. Interestingly, the temporal profile of ROS amount in U266 cells treated with NCH-51 appeared to be in a concave shape indicating a gradual suppression of ROS accumulation within the initial 4 h and subsequent induction of ROS (Figure 3a), whereas the temporal profile with SAHA continuously declined over time. Similar trends were observed with other cell lines (data not shown). As summarized in Table 2, SAHA was more effective in reducing ROS than NCH-51, and the difference in ROS amount between NCH-51 and SAHA was most evident at 24 h, when the difference in growth inhibitory effect could be observed (Figure 1). These results suggested a possibility that dynamic state of ROS in each cell could be attributed to the growth inhibitory effect of HDACi. We thus examined whether NAC, a small-molecule antioxidant compound, could modulate the effect of NCH-51 (Figure 3b). Expectedly, when U266 cells were pretreated with NAC, the NCH-51-mediated cell growth inhibition was abolished. Similar effect was observed in SAHA-treated cells as much as in NCH-51-treated cells (data not shown). These results indicated that high amount of ROS might be necessary for the induction of NCH-51-mediated cytotoxicity.

Discussion

There have been accumulating reports of successful inhibition of cancer cell growth using small-molecule HDACi compounds.^{1,7} Initial studies with SAHA indicated that the mode of action of HDACi might be through upregulating the transcriptionally repressed genes during carcinogenic processes by acetylating the repressive histones.^{12–14} However, recent reports have demonstrated that HDACi compounds also exert anticancer effects through acetylation of non-histone substrates.^{15–19} For example, HDAC6, a microtubule-associated deacetylase, was shown to be responsible for transportation of misfolded proteins to aggresome thus promoting protein degradation.^{19,29} Thus, we have attempted to clarify the mechanism of anticancer effects of NCH-51 by examining both mRNA and protein levels.

In this study, we noticed that NCH-51 induced apoptosis in sensitive cell lines greater than SAHA (Figures 1 and 2a and Table 1). Analyses of mRNA expression levels in NCH-51- and SAHA-treated cells has revealed that transcriptional repression of antiapoptotic genes, such as *survivin*, *bcl-w* and *c-FLIP* and

upregulation of cell cycle regulators, such as *p21* and *p19*, could be attributable to the induction of apoptosis and cell cycle arrest, respectively (Supplementary Table 1, 2 and Supplementary Figure 1), supporting the previous findings by others.^{12–14} There was no difference in the mRNA expression level between NCH-51- and SAHA-treated samples (Supplementary Figure 1). We have confirmed no difference in inducing activity for acetylation of histone H4 between these two HDACi's (Supplementary Figure 2). These findings suggest that NCH-51 and SAHA similarly affect gene expression presumably through histone acetylation. We thus examined the effects of these compounds on protein expression profile to understand the difference between NCH-51 and SAHA at the post-transcriptional level.

Interestingly, our proteome analyses revealed the upregulation of some antioxidant molecules including PRDX1, PRDX2 and GSTP1-1 (Table 2 and Figure 2). In addition, previous reports indicated that some other proteins identified by the present proteome analysis (Table 2) could be activated by oxidative stress.^{30–34} For example, NDPKA is reported to be activated by ROS and protected the cell from ROS-induced apoptosis.^{30,31} CLIC1 protein contains a redox-active site and is activated during ROS-triggered apoptosis.^{32,33} These findings suggested a possibility that NCH-51 might induce the cytotoxic effect by modulating the intracellular ROS content. In fact, pretreatment with NAC abolished the growth inhibitory effect of NCH-51 (Figure 3b). These results support the previous findings by others, which showed the ROS accumulation by HDACi.^{14,34,35} However, in contrast to the previous reports, both NCH-51 and SAHA downregulated the ROS content after 24 h treatment in most of the cell lines tested except for KM5 cells, in which ROS was increased by the treatment with SAHA or NCH-51 (Table 3). These findings were reproducibly observed. Although we currently do not know the reason why there was a discrepancy between our study and others, we think the time-point and/or cell characteristics may be different between them. When temporal profiles of ROS accumulation were examined (Figure 3a), we found a typical bimodal kinetics of the intracellular ROS content after treatment with NCH-51; an initial downregulation and subsequent upregulation of ROS. On the other hand, SAHA did not follow the similar kinetics and the intracellular ROS level gradually and continuously decreased (Figure 3a). Therefore, it is possible that apparent differences in the ability of cell growth inhibition between NCH-51 and SAHA, may be explained through the different effects on the redox status of cells and induction of antioxidant proteins. Interestingly, our proteome/transcriptome analyses have revealed that the upregulation of antioxidant molecules occurred at either protein or mRNA levels: GSTP1-1 was upregulated by

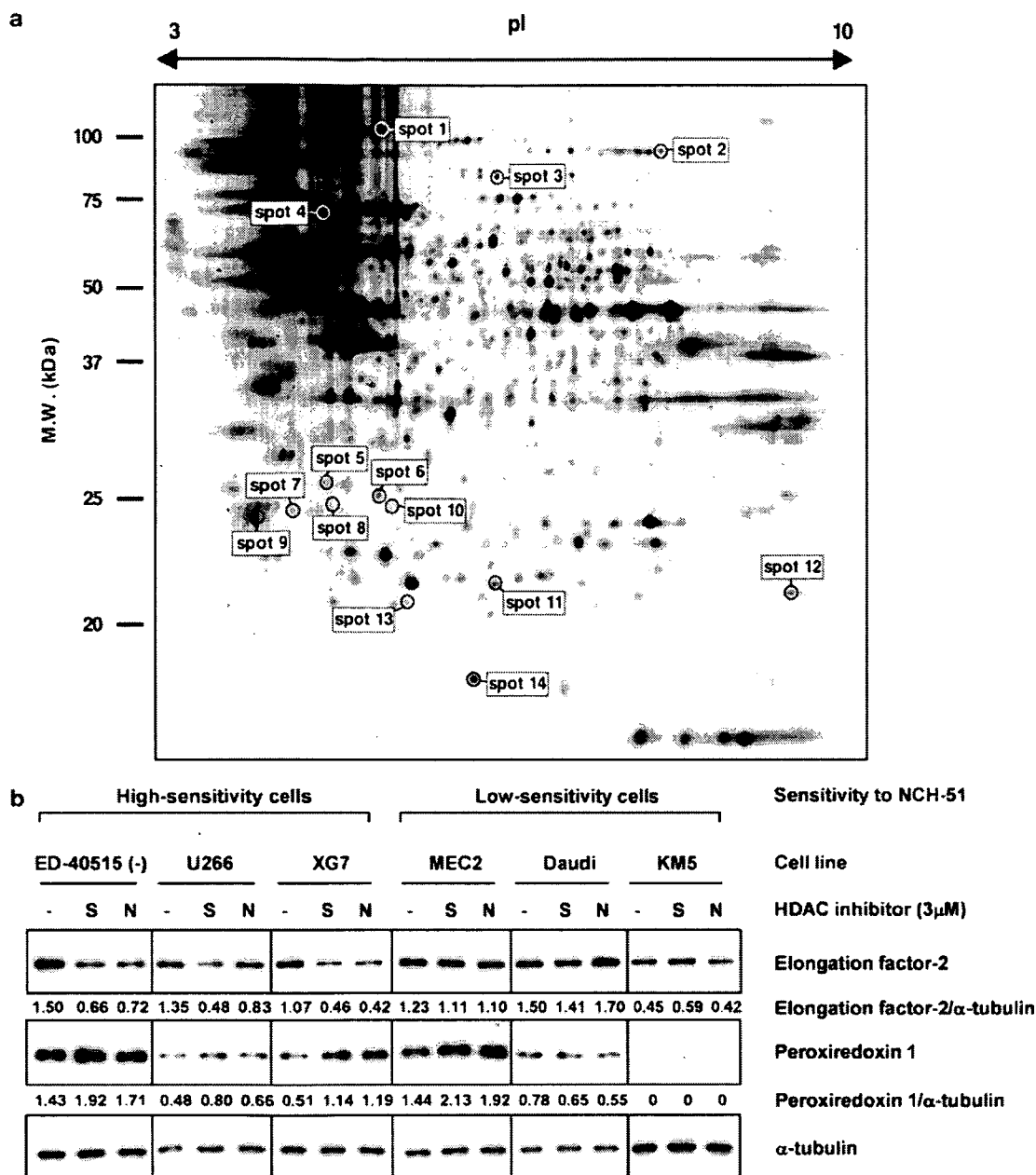


Figure 2 Proteome analysis of the effects of NCH-51. (a). 2D electrophoresis image of whole cell proteins prepared from U266 cells. After U266 cells were treated with or without 3 μ M NCH-51 for 18h, the whole cell extracts were prepared and applied to 2D electrophoresis in quadruplicates. Fourteen spots, whose relative amounts were either increased or decreased by the treatment with NCH-51 in all four gels, were indicated. Each spot number in the image corresponds to those in Table 2. MW, molecular weight (kDa). pI, isoelectric point. (b) Downregulation of elongation factor-2 (EF-2) and upregulation of peroxiredoxin 1 (PRDX1) by HDACi. The cells were treated with suberoylanilide hydroxamic acid (S) or NCH-51 (N) (3 μ M) for 18 h. Whole cell extracts were prepared and subjected to immunoblots with the indicated antibodies. Each value means the ratio of the protein amount to α -tubulin (internal control).

NCH-51 only at the protein level, and PRDX1 and PRDX2 were upregulated primarily at the transcriptional level. These findings suggest that there may be two consequent antioxidative mechanisms by which HDACi modulate ROS accumulation: (i) the mRNA level, acting through induction of transcription of antioxidant molecules *de novo*, and (ii) the protein level, which was presumably caused by blocking the cellular protein transport/degradation pathway involving aggresome and proteasome. Importantly, we have observed that SAHA but not NCH-

51 could induce acetylations of α -tubulin and HSP90 (Supplementary Figure 2), suggesting that SAHA might prolong the latter mechanism through blocking the degradation of antioxidant molecules. In fact, SAHA seemed to increase protein expression of PRDX1 greater than NCH-51 (Figure 2b). Since NCH-51 does not retain antioxidant molecules at the protein level, it induces the accumulation of ROS. Cellular levels of antioxidant molecules have been reported to be associated with the sensitivity to conventional anticancer agents.³⁶ Therefore,

Table 2 Upregulated/downregulated proteins after the treatment with NCH-51 in U266 cells

Spot no.	Protein ID	Protein name	MW	pI	Protein ratio (T/C) ^a	mRNA ratio (T/C) ^a
1	NP_001596	Alanyl-tRNA synthetase (AARS)	1 06 734	5.31	0.80	0.74
2	NP_001952	Elongation factor-2 (EF-2)	95 146	6.42	0.82	1.23
3	NP_006830	Mitochondrial inner membrane protein (IMMT)	83 626	6.08	0.83	0.64
4	NP_006588	Heat-shock 70 kDa protein 8 (HSPA8)	70 854	5.28	0.82	1.18
5	NP_001279	Cl ⁻ intracellular channel protein 1 (CLIC1)	26 775	5.09	1.34	0.67
6	NP_002809	Protease activator 28 β subunit (PA28β)	27 213	5.44	1.54	1.03
7	NP_004300	Rho GDP-dissociation inhibitor 1 (Rho GDIα)	23 193	5.02	1.42	0.92
8	NP_002779	Proteasome subunit α type3	28 284	5.19	1.32	0.73
9	NP_663723	14-3-3 ζ/δ (PKC inhibitor protein 1)	28 828	4.73	1.37	0.83
10	NP_039234	Cl intracellular channel protein 4 (CLIC4)	28 754	5.45	1.38	0.86
11	NP_000843	Glutathione S-transferase P 1-1(GSTP1-1)	23 210	5.44	1.38	0.98
12	NP_002565	Peroxiredoxin 1 (thioredoxin peroxidase 2) (PRDX1)	22 096	8.27	1.30	1.4
13	NP_005800	Peroxiredoxin 2 (thioredoxin peroxidase 1) (PRDX2)	21 747	5.67	1.30	1.35
14	NP_000260	Nucleoside diphosphate kinase A (NDPKA)	17 138	5.83	1.50	1.04

^aThe results are indicated as the ratio of NCH-51-treated sample to untreated control (T/C).

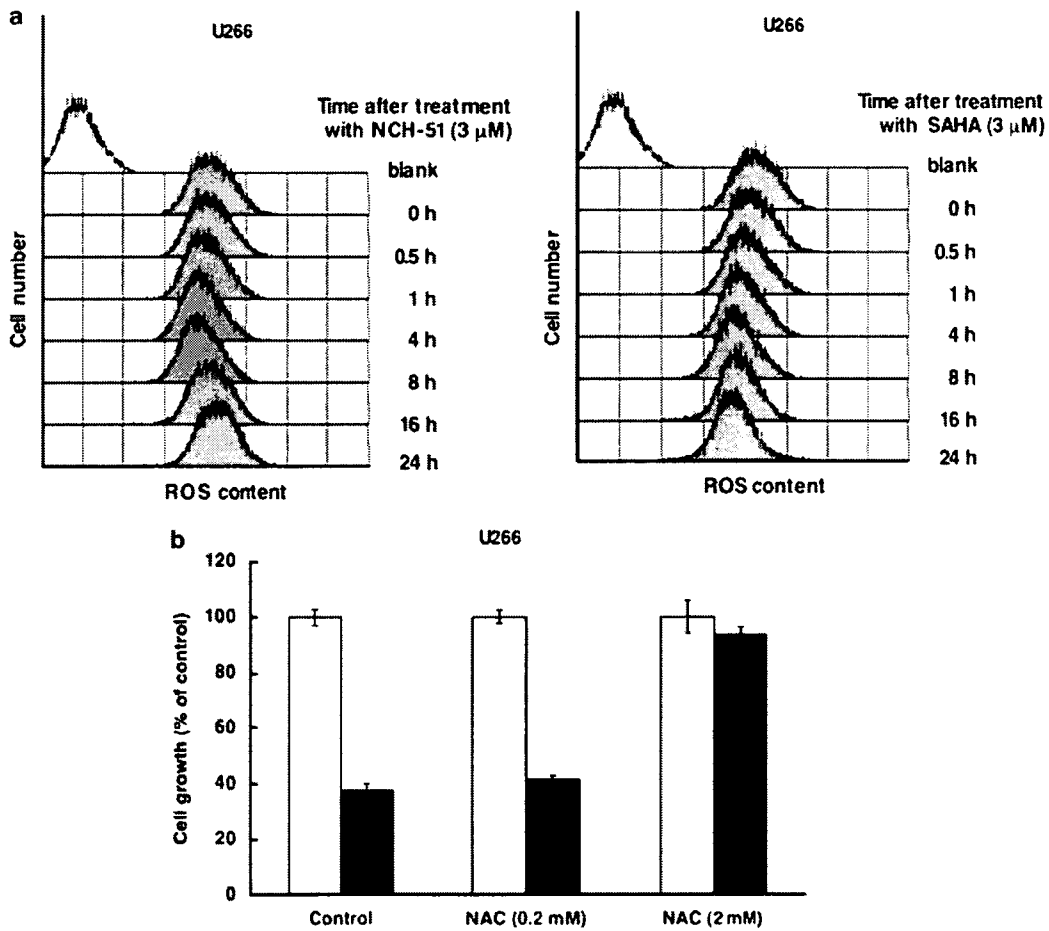


Figure 3 Effects of NCH-51 and suberoylanilide hydroxamic acid (SAHA) on reactive oxygen species (ROS) accumulation. (a) Time course of intracellular ROS after the treatment with HDACi. U266 cells were treated with NCH-51 or SAHA (3 μM) given the indicated time (0–24 h). After the treatment, H₂-DCFDA was added and further incubated for 30 min. ROS content was measured by flowcytometry. Blank, H₂-DCFDA-untreated control. (b) Effects of *N*-acetyl-L-cysteine (NAC) on the NCH-51-induced cell growth inhibition. U266 cells were treated with or without NCH-51 (3 μM) for 24 h in the presence or absence of NAC (0.2 or 2 mM). Cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Open and closed bars indicate untreated and the NCH-51-treated cells. The results are shown as the percentage compared to untreated control. Experiments were done in triplicates and the mean values ±s.d. are shown.

NCH-51 can not only induce apoptosis through ROS accumulation, but also enhance the cytotoxicity of other agents in the combination treatment more efficiently than SAHA.

Our proteome analysis has also identified several proteins other than antioxidant molecules, including EF-2, AARS and HSPA8. We confirmed that EF-2 protein, a member of the GTP-

Table 3 ROS content and growth inhibitory effect after the treatment with HDAC inhibitor

Cell line	ROS content (% of control) ^a		Growth inhibition (% of control) ^b	
	NCH-51 (3 μM)	SAHA (3 μM)	NCH-51 (3 μM)	SAHA (3 μM)
Jurkat	86	47	63.5 ± 0.8	37.7 ± 2.5
MT-2	100	100	38.8 ± 0.3	11.0 ± 2.3
ED-40515 (-)	82	56	71.2 ± 1.2	44.1 ± 1.0
MEC2	59	46	41.3 ± 1.1	3.5 ± 2.8
MO1043	91	65	46.0 ± 0.5	7.2 ± 2.9
Daudi	74	54	25.6 ± 1.8	15.1 ± 3.0
U266	95	85	80.7 ± 1.8	51.7 ± 3.2
XG7	74	28	66.5 ± 1.1	23.1 ± 1.6
KM5	126	119	20.5 ± 0.5	14.6 ± 1.2
ILKM-2	94	94	85.5 ± 0.2	81.3 ± 1.1

Abbreviations: HDAC, histone deacetylase; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid.

^aEach value shows the average of 20 000 cells counted.

^bEach value shows the mean ± s.d.

binding translational elongation factor family,³⁷ was specifically decreased in high-sensitivity cell lines such as ED-40515 (-) and U266 cells (Figure 2b) after 16 h treatment (data not shown). However, gene expression of EF-2 was upregulated after the treatment with NCH-51 (Table 2). This discrepancy indicates a possibility that NCH-51 could induce rapid turnover of EF-2 protein followed by upregulation of RNA expression. Similar effects on EF-2 were also observed with SAHA (Figure 2b). Since EF-2 has been reported to be inactivated by ROS and lead to inhibition of translation,³⁸ it is suggested that locally induced ROS by HDACi might induce EF-2 inactivation and degradation presumably by direct oxidation. Although the mechanism by which EF-2 is downregulated by HDACi remains unclear, EF-2 may be used as a feasible surrogate marker to evaluate the susceptibility of HDACi. Similar to EF-2, AARS and HSPA8 were downregulated at the protein level. It is known that HSPA8 are involved in protein folding and transport.³⁹ Thus, these findings collectively suggest that NCH-51 might arrest protein synthesis and transportation.

In conclusion, our study demonstrates the therapeutic advantage of NCH-51 on growth inhibition of lymphoid malignant cells. Importantly, NCH-51 did not affect the cell growth of normal PBMCs with the effective concentrations on malignant cells (Figure 1b). In addition to its therapeutic efficacy and selectivity, NCH-51 has additional advantages in clinical use based on its pharmacological features. Therefore, NCH-51 could be a useful anticancer agent against lymphoid malignancies.

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Efficient identification and quantification of proteins using isotope-coded 1-(6-methylnicotinoyloxy)-succinimides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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We describe a convenient and useful method for the identification and relative quantification of proteins using light and heavy reagents, 1-(6-methylnicotinoyloxy)succinimides (6-CH₃-Nic-NHS and 6-CD₃-Nic-NHS, respectively). This method is based on the chemical derivatization of amino groups of tryptic peptides with these reagents, i.e., the basic moiety of the reagents thus incorporated into both the N-terminal amino group and the ϵ -amino group of the lysine residue would improve the ionization efficiency of tryptic peptides. An increase in protein sequence coverage is achieved by derivatization with these reagents or by combination of mass values before and after derivatization. Since a combination of 6-CH₃-Nic-NHS and d₃-labeled reagent (6-CD₃-Nic-NHS) generates a 3 Da mass difference per reaction site, the d₃-labeled reagent shifts the mass values of d₀-labeled peptides according to the number of reactive amino groups in the peptides. In the case of tryptic peptides, the mass values of C-terminal arginine and lysine peptides are shifted by 3 and 6 Da, respectively. Further, the 3 Da mass difference between 6-CH₃-Nic-NHS and 6-CD₃-Nic-NHS offers a means for the relative quantification of protein by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Copyright © 2007 John Wiley & Sons, Ltd.

Mass spectrometry (MS) has been successfully used for the identification of proteins. Peptide mass fingerprinting (PMF) by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) is one of the most widely used methods for protein identification. The mass values of tryptic peptides derived from protein digestion are matched to those of theoretical peptides by database search such as MASCOT. To identify the parent protein with high accuracy and confidence, the number of matched tryptic peptides is important. Park *et al.* have reported the dansylation of tryptic peptides for protein identification.¹ Sequence coverage and protein identification score were improved by combining mass data obtained before and after dansylation. The ionization efficiency of arginine-containing peptides is higher than that of lysine-containing peptides.² Based on these results, guanidination^{3–7} or amidination^{8,9} of the ϵ -amino group of lysine residues was used for signal enhancement of lysine-containing peptides and for peptide quantification.^{7,8} Wa *et al.* examined experimental factors such as the type of matrix and the protein digestion method

to obtain high sequence coverage in MALDI-TOF MS using human serum albumin as the model protein.¹⁰

Recently, MS-based quantification was performed by stable-isotope labeling of proteins or tryptic peptides.¹¹ Many methods have been developed for the incorporation of stable isotopes. Metabolic labeling using labeled nutrients,¹² chemical labeling of proteins¹³ or peptides,^{14–19} using isotope-coded reagent, and enzymatic labeling²⁰ are widely used. In the chemical labeling method, isotope-coded reagents target reactive sites on a protein or peptide, such as the sulfhydryl group of cysteine,^{14,15} N-terminal amine,^{16,17} ϵ -amino group of lysine,^{7,8} tryptophan,¹⁸ and the C-terminal carboxyl group.¹⁹ The isotope-coded affinity tag (ICAT) method reported in 1999 by Gygi *et al.*¹⁴ is the most widely used. In that method, the sulfhydryl group of cysteine is selectively modified with a biotin-conjugated reagent prior to protease treatment. As only cysteine-containing peptides are labeled and isolated, peptide mixtures are less complex. However, for proteins having no cysteine residues, the ICAT method is not applicable. Further, the large size of the ICAT reagent increases the complexity of tandem mass spectral (MS/MS) measurements because it is fragmented as well. Chemical modification methods targeting amino groups

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have been extensively developed for the identification and quantification of proteins. In their study published in 2000, Münchbach *et al.* protected the ϵ -amino group of lysines by succinylation and labeled N-terminal amines of tryptic peptides with 1-(d_0/d_4 -nicotinoyloxy)succinimide (d_0/d_4 -Nic-NHS).¹⁶ In 2003, we reported the syntheses of 1-(6-methylnicotinoyloxy)succinimide (6- CH_3 -Nic-NHS), 1-(2,6-dimethylnicotinoyloxy)succinimide (2,6-di CH_3 -Nic-NHS), and their d -labeled analogues (6- CD_3 -Nic-NHS and 2,6-di CD_3 -Nic-NHS), and showed that these reagents can be prepared easily and at a low cost.²¹ In 2005, Schmidt *et al.* reported the isotope-coded protein label (ICPL) strategy in which d_0 - or d_4 -Nic-NHS was used to label proteins.¹³ A method that has recently gained popularity is the isobaric 'tag for relative and absolute quantification' (iTRAQ) reported in 2004 by Ross *et al.*¹⁷ In this approach, four reagents are used and the labeled peptides have the same mass value in MS, but the fragment ions derived from the reagents facilitate quantification in MS/MS. Moreover, this multiplex strategy allows measurement of four samples in a single analysis. The conversion of the ϵ -amino group of lysine into homoarginine (guanidination) has also two advantages for PMF-based methods.²⁻⁷ First, it increases the sensitivity of lysine-containing peptides and, second, the C-terminal amino acids of tryptic peptides are identified.

N-Hydroxysuccinimidyl (NHS) esters are the most commonly used reagents for chemical modification of the amino groups of peptides^{16,17} and proteins.¹³ For the efficient modification of N-terminal amino groups, the derivatization is performed at high pH. However, at high pH, selectivity is lost and partial derivatization of the ϵ -amino groups of lysine residues and the hydroxyl groups of tyrosine residues also takes place. For the relative quantification of peptides and proteins by the chemical modification method using isotope-coded reagents, the native peptides should be derivatized by the reagents selectively and efficiently. However, it is difficult to derivatize only the N-terminal amino groups of peptides by pH control. Münchbach *et al.* protected the ϵ -amino groups of lysine residues by succinylation and selectively derivatized the N-terminal amino

groups by 1-(d_0/d_4 -nicotinoyloxy)succinimide (d_0/d_4 -Nic-NHS) after digestion with Glu(Asp)C-protease (V8).¹⁶

Compounds **1** and **2** shown in Fig. 1 are amine-reactive reagents that we previously reported.²¹ The reactivities of these two reagents for the amino group and the ionization potentials of peptides derivatized with these reagents are considered to be the same, because only the hydrogen in the methyl group is replaced with the isotope atom, deuterium. The molecular weight of reagent **2** differs from that of reagent **1** by 3 Da. This mass difference is useful for the analysis of peptide sequences (number of amino group) and the relative quantification of proteins by MS.

Our approach involves the complete derivatization of both the N-terminal amino group and the ϵ -amino group of lysine residues of tryptic peptides with the reagents, as shown in Fig. 1. In this study, first, the conditions for derivatization were optimized and the effect of the derivatization on the MALDI-TOF MS signal intensity was studied using model peptides and tryptic peptides of model proteins. Next, tryptic peptides of both a single protein and five-protein mixture were used to demonstrate the applicability of our method to protein identification. The dynamic range of the relative quantification was determined using a binary mixture containing **1** (d_0)- and **2** (d_3)-derivatized peptides of tryptic myoglobin. Finally, our method was applied to the relative quantification of a mixture of five proteins.

EXPERIMENTAL

Chemicals

Bovine serum albumin (BSA), myoglobin from horse heart, cytochrome *c* from horse heart, α -lactalbumin from bovine milk, lysozyme from chicken egg white, iodoacetamide (IAA), α -cyano-4-hydroxycinnamic acid (CHCA), ammonium bicarbonate, and trypsin (proteomics grade) were purchased from Sigma (St. Louis, MO, USA). DL-Dithiothreitol (DTT) and triethylammonium bicarbonate buffer (TEAB) were purchased from Fluka (St. Louis, MO, USA). HPLC grade acetonitrile, 10% ammonium hydroxide (NH_4OH), and trifluoroacetic acid (TFA) were purchased

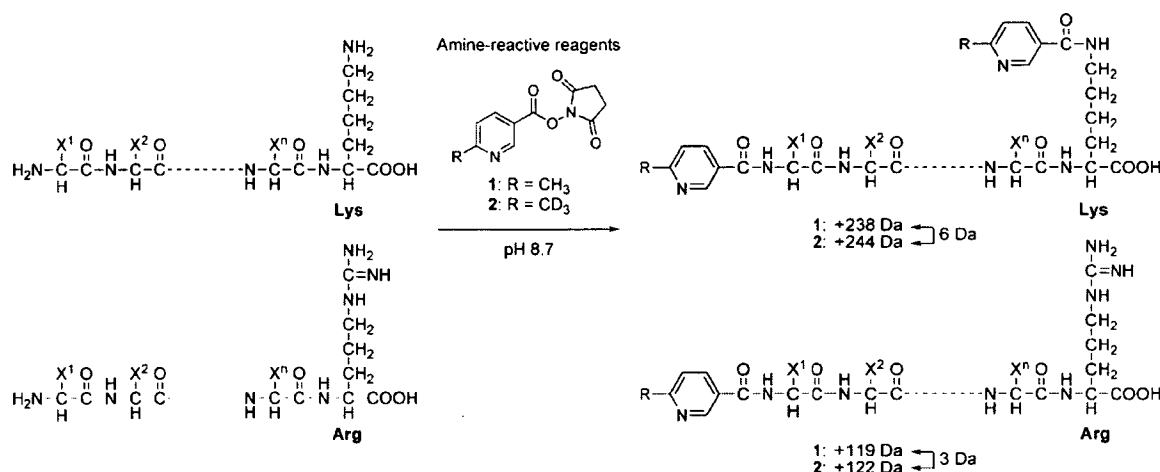


Figure 1. Chemical structures of amine-reactive reagents and scheme for derivatization. Peptides containing C-terminal lysine and arginine are derivatized with two and one molecules of reagent, respectively.

from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bradykinin (RPPGFSPFR, $[M+H]^+ = 1060.57$) was purchased from Peptide Institute, Inc. (Osaka, Japan). Synthetic peptides RPPGFpSPFR ($[M+H]^+ = 1140.54$), TNASYSYSPRAK ($[M+H]^+ = 1094.56$), and TNAPSYSPRAK ($[M+H]^+ = 1174.53$) were custom made by Sigma-Aldrich Japan (Tokyo, Japan). Amine-reactive reagents **1** and **2** shown in Fig. 1 were prepared by us as reported previously.²¹

Derivatization of model peptides

Method 1

A labeling reagent was dissolved in acetonitrile at a concentration of 50 mM. A model peptide was dissolved in water at a concentration of 1 mM. The model peptide solution (2 μ L) was mixed with 50 mM NH_4HCO_3 (pH 7.8, 2 μ L) or 50 mM TEAB (pH 8.7, 2 μ L). To this peptide solution was added 20 μ L of the reagent solution. The reaction mixture was mixed with a vortex mixer for 10 s and left at room temperature for 0.5–2 h. Then, it was quenched by adding 176 μ L of 0.1% TFA (10 pmol/ μ L).

Method 2

A labeling reagent was dissolved in acetonitrile at a concentration of 100 mM. A model peptide was dissolved in water at a concentration of 5–200 pmol/ μ L. The model peptide solution (10 μ L) was mixed with 100 mM TEAB (pH 8.7, 10 μ L). To this peptide solution was added the reagent solution (20 μ L), and the reaction mixture was stirred at room temperature for 1 h. Then, 20 μ L of 10% NH_4OH was added and the mixture was stirred at room temperature for 2 h. The reaction was quenched by adding 10% TFA (100 μ L) and the mixture was diluted with water (40 μ L) to give a peptide solution of 0.25–10 pmol/ μ L.

Tryptic digestion of proteins

Stock solutions of proteins were prepared in 50 mM NH_4HCO_3 at a concentration of 1.5 μ g/ μ L. Trypsin was dissolved in 1 mM HCl at a concentration of 1 μ g/ μ L. Prior to tryptic digestion, the disulfide bonds of proteins were reduced with DTT and subsequently alkylated with IAA, as follows. A protein solution (100 μ L) was added to a mixture of 50 mM NH_4HCO_3 (150 μ L) and 200 mM DTT (20 μ L) and the mixture was incubated at 95°C for 10 min. To the reduced sample was added 200 mM aqueous IAA (30 μ L), and the mixture was stored in the dark at room temperature for 30 min. Trypsin digestion was performed by adding 3 μ L of trypsin solution to 300 μ L of reduced/carbamidomethylated solutions (1:50 w/w). The mixture was incubated at 37°C for 14 h. The digested sample (0.5 μ g/ μ L) was stored at –30°C.

Derivatization of tryptic peptides

The labeling reagent was dissolved in acetonitrile at a concentration of 100 mM. Then 10 μ L of the digested sample (0.5 μ g/ μ L) was mixed with 10 μ L of 100 mM TEAB (pH 8.7). To this solution was added 20 μ L of the reagent solution and the mixture was stirred at room temperature for 1 h. Then, 20 μ L of 10% NH_4OH was added and the mixture was stirred at room temperature for 2 h. The reaction was quenched by

adding 80 μ L of 10% TFA and the mixture was diluted with 60 μ L of water.

MALDI-TOF MS analysis

MALDI-TOF mass spectra were acquired on an AXIMA-CFR instrument (Shimadzu / Kratos, Kyoto, Japan). The operating conditions were as follows: nitrogen laser (337 nm); reflection mode; detection of positive ions; acceleration voltage, 20 kV. Each spectrum was obtained by accumulating 50 shots. High-purity CHCA (proteomics grade) was used as the MALDI matrix throughout this study without further purification. CHCA was dissolved in 50% acetonitrile in 0.05% TFA at a concentration of 10 mg/mL. Samples for MS analysis were prepared by mixing 0.5 μ L of the reaction mixture with 0.5 μ L of the matrix solution on the MALDI target plate. The applied samples were allowed to dry at room temperature before transferring into the mass spectrometer.

Database search

The PMF search was carried out using MASCOT with the NCBI database. The search parameters were as follows: Taxonomy, metazoan (animals); enzyme, trypsin; fixed modifications, carbamidomethyl (C), 6- CH_3 -Nic (N-term and K); variable modifications, none; peptide tolerance, ± 0.2 Da; mass values, $[M+H]^+$ and monoisotopic; and missed cleavages, 1.

RESULTS AND DISCUSSION

Derivatization of model peptides

To optimize the derivatization conditions, bradykinin (RPPGFSPFR, $[M+H]^+ = 1060.57$) and three synthetic peptides, RPPGFpSPFR ($[M+H]^+ = 1140.54$), TNASYSYSPRAK ($[M+H]^+ = 1094.56$), and TNAPSYSPRAK ($[M+H]^+ = 1174.53$), were used as model peptides. The reasons why these peptides were used are that the C-terminal amino acid of the tryptic digest is arginine or lysine, and the phosphorylated peptide generally has low ionization efficiency in the positive ion mode due to the negatively charged phosphate group. The derivatization with reagent **1** was performed using 50 mM NH_4HCO_3 (pH 7.8) and 50 mM TEAB (pH 8.7) at room temperature for 0.5–2 h (method 1), and the MALDI mass spectra of the derivatized peptides are shown in Fig. 2. Figures 2(a)–2(c) show the MALDI mass spectra of RPPGFSPFR, RPPGFpSPFR, and TNASYSYSPRAK derivatized with reagent **1** at pH 7.8 for the indicated times, respectively. Under these conditions, no signals of the native peptide were observed in all cases. In the MALDI mass spectra of peptides containing C-terminal arginine, only the signal of a mono-derivatized peptide was observed at m/z 1179.68 (*) and 1259.61 (*), as shown in Figs. 2(a) and 2(b), respectively. In contrast, in the MALDI mass spectrum of the peptide containing C-terminal lysine, two signals corresponding to mono-derivatized and di-derivatized peptides were observed at m/z 1213.43 (*) and 1332.46 (**), as shown in Fig. 2(c). This is because two amino groups exist at both the N-terminal amino acid and the ϵ -position of lysine. On the other hand, Figs. 2(d)–2(f) show the MALDI mass spectra after derivatization at pH 8.7. Under this high pH condition,

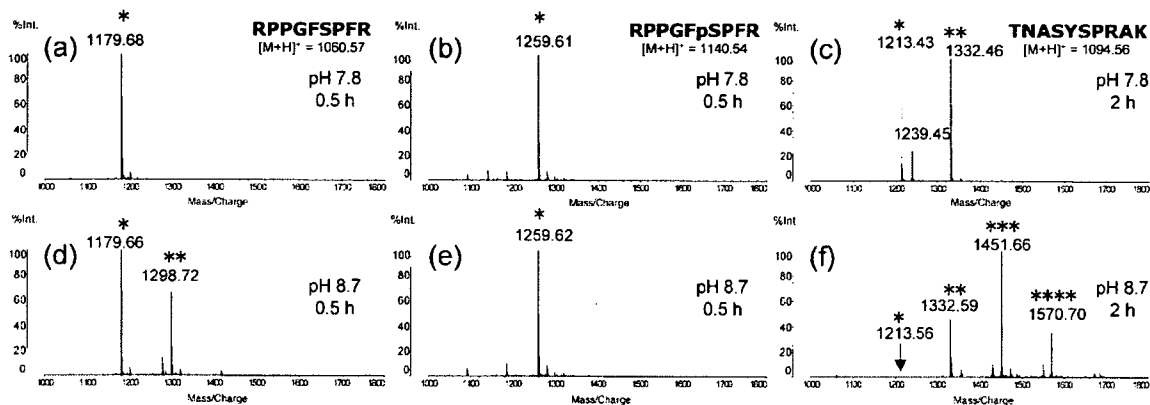


Figure 2. Comparison of reactivity of reagent 1 toward amino groups of peptides at pH 7.8 and pH 8.7. MALDI mass spectra of model peptides derivatized with reagent 1 are shown: (a) RPPGFSPFR, pH 7.8, 0.5 h; (b) RPPGFpSPFR, pH 7.8, 0.5 h; (c) TNASYSRAK, pH 7.8, 2 h; (d) RPPGFSPFR, pH 8.7, 0.5 h; (e) RPPGFpSPFR, pH 8.7, 0.5 h; and (f) TNASYSRAK, pH 8.7, 2 h. Number of asterisks (*) indicates the number of labels introduced into the peptide by the derivatization. A volume of 5 pmol of peptide was spotted on MALDI sample plate.

the derivatization of the hydroxyl groups also proceeded. Thus, in the MALDI mass spectrum of serine-containing RPPGFSPFR (Fig. 2(d)), the signal of the di-derivatized peptide that was not observed at pH 7.8 was observed at m/z 1298.72 (**). In the MALDI mass spectrum of RPPGFpSPFR shown in Fig. 2(e), the signal of the di-derivatized peptide was not observed because the hydroxyl group of the serine residue was blocked by the phosphorylation. From these results, it was concluded that, at pH 8.7, the hydroxyl group of the serine residue was also derivatized while the arginine residue was not. In TNASYSRAK, two amino groups were completely derivatized at pH 8.7, as shown in Fig. 2(f), and its MALDI mass spectrum showed no signal of a mono-derivatized peptide at m/z 1213.56 (*). On the other hand, two signals corresponding to tri-derivatized and tetra-derivatized peptides were further observed at m/z 1451.66 (***) and 1570.70 (****). These two signals may be due to derivatization of the hydroxyl groups of the two serine

residues rather than derivatization of the N-terminal amino group and the ϵ -amino group of lysine residue. To use the amine-reactive reagents for quantitative analysis, the reagents should be incorporated only into the N-terminal amino group of a peptide or both the N-terminal amino group and the ϵ -amino group of the lysine residue. However, as described above, it is difficult to derivatize only the N-terminal amino group by pH control. From these results, we devised the following approach. First, both the N-terminal amino group and the ϵ -amino group of lysine residue were completely derivatized at pH 8.7 although some esterification of hydroxyl groups of tyrosine, serine, and threonine proceeded. Second, the esterification site was cleaved off by hydrolysis with ammonium hydroxide. Figure 3 shows the MALDI mass spectra of the model peptides before (Figs. 3(a) and 3(b)) and after (Figs. 3(c) and 3(d)) derivatization with reagent 1 using method 2. Figure 3(c) shows the MALDI mass spectrum of bradykinin (RPPGFSPFR, $[M+H]^+ = 1060.57$)

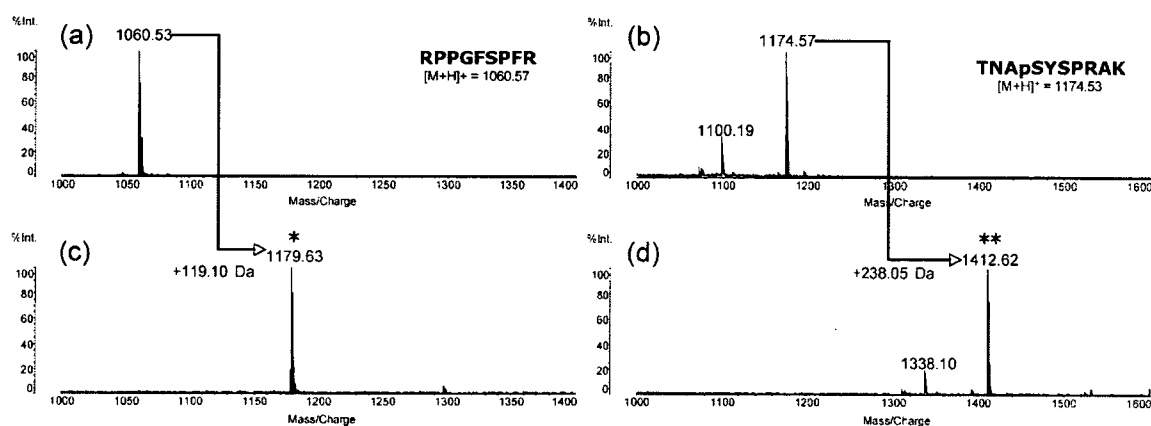


Figure 3. MALDI spectra of model peptides before and after derivatization. (a, b) MALDI spectra of 125 fmol of bradykinin (RPPGFSPFR) and 1.25 pmol of synthetic phosphorylated peptide (TNApSYSRAK), respectively. (c, d) MALDI spectra of 125 fmol of bradykinin and 1.25 pmol of the phosphorylated peptide after derivatization with reagent 1. Number of asterisks (*) indicates the number of labels introduced into the peptide by the derivatization. Signals at m/z 1100.19 in (b) and m/z 1338.10 in (d) are due to impurities in the sample.

after derivatization. The mass increase (+119 Da) of the derivatized bradykinin (m/z 1179.63) corresponded to a mono-adduct at the N-terminal amino group. On the other hand, Fig. 3(d) shows the MALDI mass spectrum of the synthetic phosphorylated peptide (TNApSYSYSPRAK, $[M+H]^+ = 1174.53$) after derivatization. The mass increase (+238 Da) of the derivatized phosphorylated peptide (m/z 1412.62) corresponded to di-adducts. It is clear that the derivatization was completed at both the N-terminal amino group and the ϵ -amino group of the C-terminal lysine residue, because no signals of native, partially derivatized or multiply derivatized peptides were observed.

Derivatization of tryptic peptides of model proteins

We used myoglobin^{5,16} as the model protein to demonstrate the applicability of our amine-derivatization method. Because myoglobin contains 19 lysines and 2 arginines, tryptic digestion of it generates many lysine-containing peptides.

The results of tryptic digestion of myoglobin are shown in Fig. 4 and Table 1. Figure 4(a) shows the MALDI mass spectrum of the digest without derivatization and Figs. 4(c) and 4(e) show those of the tryptic peptides derivatized with reagents 1 (d_0) and 2 (d_3), respectively. Figures 4(b), 4(d) and 4(f) show the MALDI mass spectra of Figs. 4(a), 4(c) and 4(e) in the low-mass range (m/z 400–700), respectively. Figure 4(g) shows the difference in sequence coverage of

myoglobin with (underlined) and without (**bold**) derivatization. The peptide sequences of the detected peaks were assigned by MASCOT search and are summarized in Table 1 (matched mass values are shown in **bold**). Each peak in Fig. 4 was marked with an entry number shown in Table 1.

Before derivatization, the number of matched peptides was eight (9, 11–17) and sequence coverage was 62% by MASCOT search, as shown in Fig. 4(g) in bold. On the other hand, as shown in Fig. 4(c), peptides 9, 11–14, 16, and 17 were completely derivatized with reagent 1 and neither partially nor multiply derivatized signals were observed. The labeling efficiency of our reagents was almost the same as that of others reported.¹⁶ Under these reaction conditions, no signals of derivatized peptide 15 were observed. It was evident that the signal intensities of peptides 9, 11, 13, and 16 were improved by the derivatization (9*, 11**, 13**, and 16***) when compared with their signal intensities using that of peptide 14* as standard. The number of asterisks (*) indicates the number of labels introduced into the peptide by the derivatization. Three peptides (7**, 8***, and 10***) could be observed and matched by MASCOT search only after derivatization. After derivatization, the number of matched peptides was ten (7–14, 16, and 17) and sequence coverage was slightly increased to 64%, as shown in Fig. 4(g) with underlining. The minimal increase of the sequence coverage may be due to loss of high-mass peptide 15. However, by using both native and derivatized peptides for database search, the sequence coverage was increased from 62% to

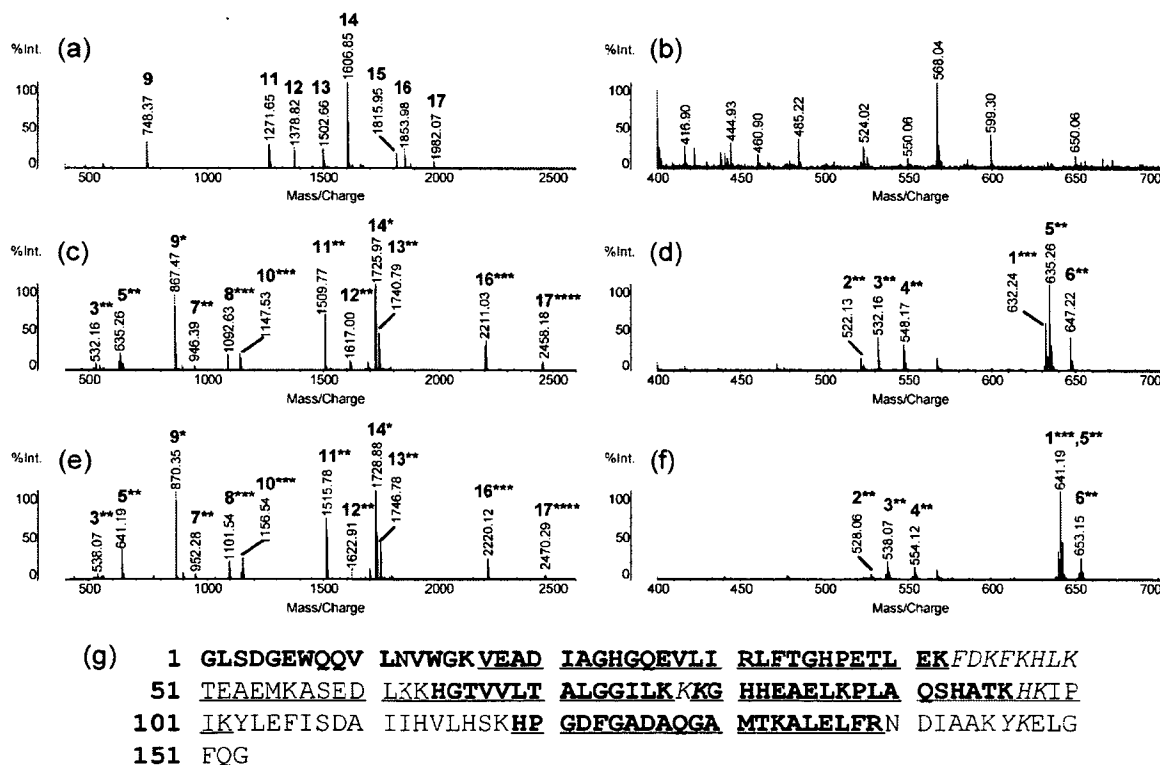


Figure 4. MALDI mass spectra of tryptic myoglobin: (a, b) without derivatization; (c, d) with derivatization by reagent 1 (d_0); and (e, f) with derivatization by reagent 2 (d_3). (b), (d), and (f) are enlarged spectra of (a), (c), and (e), respectively. Peaks are marked with entry numbers shown in Table 1. Number of asterisks (*) indicates the number of labels introduced into the peptide by the derivatization. Each MALDI spot contained 12.5 ng (0.7 pmol) of myoglobin. (g) Sequence coverage in myoglobin before (**bold**) and after (underlined) derivatization.

Table 1. Sequences and mass values of myoglobin tryptic peptides

No.	Start-End	Native		Sequence	d_0 -Labeled		d_3 -Labeled		Delta
		Observed [M+H] ⁺	Mr(calc) M		Mr(calc) M	Observed [M+H] ⁺	Observed [M+H] ⁺		
1	78-79		274.20	K*K'	631.32	632.24	641.19	9	
2	97-98		283.16	HK'	521.24	522.13	528.06	6	
3	46-47		293.17	FK'	531.25	532.16	538.07	6	
4	146-147		309.17	YK'	547.25	548.17	554.12	6	
5	48-50		396.25	HLK'	634.33	635.26	641.19	6	
6	43-45		408.20	FDK'	646.28	647.22	653.15	6	
7	51-56		707.32	TEAEMK'	945.40	946.39	952.28	6	
8	97-102		734.48	HK'IPIK'	1091.60	1092.63	1101.54	9	
9	134-139	748.37	747.43	ALELFR	866.47	867.47	870.35	3	
10	57-63		789.42	ASEDLK*K'	1146.54	1147.53	1156.54	9	
11	32-42	1271.65	1270.66	LFTGHPETLEK'	1508.74	1509.77	1515.78	6	
12	64-77	1378.82	1377.83	HGTVVLTALGGILK'	1615.91	1617.00	1622.91	6	
13	119-133	1502.66	1501.66	HPGDFGADAQQGAMTK'	1739.74	1740.79	1746.78	6	
14	17-31	1606.85	1605.85	VEADIAGHGQEVLR	1724.89	1725.97	1728.88	3	
15	1-16	1815.95	1814.90	GLSDGEWQQVNLVWGK'	2052.98	N.D.	N.D.	—	
16	80-96	1853.98	1852.95	GHHEAELK'PLAQSHATK'	2210.07	2211.03	2220.12	9	
17	79-96	1982.07	1981.05	K'GHHEAELK'PLAQSHATK'	2457.21	2458.18	2470.29	12	

N.D.: not detected.

74%. This increase of 12% is due to newly matched peptides 7^{**}, 8^{**}, and 10^{***} after derivatization, as shown in Fig. 4(g) with only underlining and no bold. From these results, it was expected that sequence coverage could be increased by a combination of mass values obtained before and after derivatization.

By derivatization of one amino group with reagents 1 and 2, the molecular weight of the derivatized peptide was increased by 119 and 122 Da, respectively, and the mass shift of the peptide derivatized with reagent 2 from that with reagent 1 was 3 Da. As shown in Fig. 4(c), signals of peptides 14, 13, 16, and 17 were observed at m/z 1725.97 (14^{*}), 1740.79 (13^{**}), 2211.03 (16^{***}), and 2458.18 (17^{****}) after derivatization with reagent 1 (d_0). These peptides contained one, two, three, and four amino groups, respectively, based on the mass increase and the sequence assignment by MASCOT search. As shown in Fig. 4(e), when reagent 2 (d_3) was used, the mass values shifted by 3 Da (14^{*}: m/z 1728.88), 6 Da (13^{**}: m/z

1746.78), 9 Da (16^{***}: m/z 2220.12), and 12 Da (17^{****}: m/z 2470.29), respectively, from the corresponding peaks shown in Fig. 4(c). These results demonstrate that the mass shift by a combination of two reagents shows the number of labels introduced into the peptide (the number of amino groups). This method is useful for the identification of the C-terminal amino acid of the tryptic digest.

Figures 4(b), 4(d) and 4(f) are enlarged spectra (m/z 400–700) of Figs. 4(a), 4(c), and 4(e), respectively. Generally, it is difficult to use MALDI-TOF MS for the analysis of small molecules ($m/z < 500$ Da) because of the presence of a variety of matrix-related ions in the low-mass range below 500 Da. However, six signals were observed after derivatization with reagent 1 (Fig. 4(d)), which corresponded to di- and tripeptides 1–6 derivatized with reagent 1, based on the mass increase. Comparing the spectrum of peptides derivatized with reagent 1 (d_0) (Fig. 4(d)) with that of ones derivatized with 2 (d_3) (Fig. 4(f)), the signals were found to be

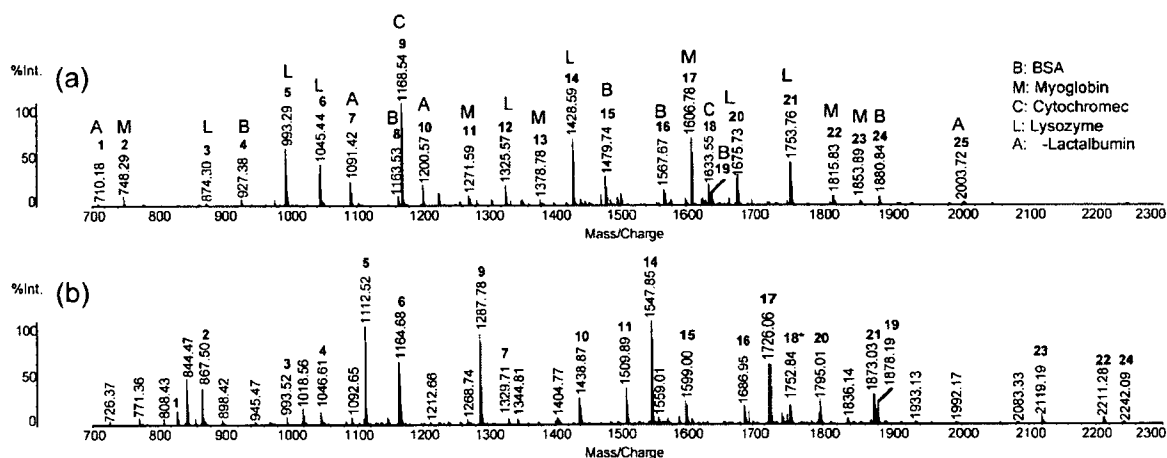


Figure 5. Derivatization of tryptic digest of five-protein mixture. (a) MALDI mass spectra of tryptic digests of a five-protein mixture. (b) MALDI mass spectrum of a mixture of tryptic digests derivatized with reagent 1. Peaks are marked with entry numbers from Table 2.

Table 2. Sequences and mass values of tryptic peptides of the five-protein mixture

No.	Native		Protein	Start-End	Sequence	Number of labels	Labeled	
	Observed [M+H] ⁺	Mr (Calc) M					Mr (Calc) M	Observed [M+H] ⁺
1	710.18	709.32	α -Lac	25-29	CEVFR	1	828.36	829.39
2	748.29	747.43	Myo	134-139	ALELFR	1	866.47	867.50
3	874.30	873.41	Lys	33-39	HGLDNYR	1	992.45	993.52
4	927.38	926.49	BSA	161-167	YLVEIAR	1	1045.53	1046.61
5	993.29	992.39	Lys	80-86	WWCNDGR	1	1111.43	1112.52
6	1045.44	1044.54	Lys	135-143	GTDVQAWIR	1	1163.58	1164.68
7	1091.42	1090.51	α -Lac	134-141	LDQWLCEK	2	1328.59	1329.71
8	1163.53	1162.62	BSA	66-75	LVNELTEFAK	2	1400.70	N.D.
9	1168.54	1167.61	Cyt	28-38	TGPNLHGLFGR	1	1286.65	1287.78
10	1200.57	1199.65	α -Lac	118-127	VGINYWLAHK	2	1437.73	1438.87
11	1271.59	1270.66	Myo	32-42	LFTGHPETLEK	2	1508.74	1509.89
12	1325.57	1324.62	Lys	40-51	GYSLGNWVCAAK	2	1562.70	N.D.
13	1378.78	1377.83	Myo	64-77	HGTVVLTALGGILK	2	1615.91	N.D.
14	1428.59	1427.64	Lys	52-63	FESNFNTQATNR	1	1546.68	1547.85
15	1479.74	1478.79	BSA	421-433	LGEYGFQNALIVR	1	1597.83	1599.00
16	1567.67	1566.74	BSA	347-359	DAFLGSFLYEYSR	1	1685.78	1686.95
17	1606.78	1605.85	Myo	17-31	VEADIAGHGQEVLR	1	1724.89	1726.06
18	1633.55	1632.76	Cyt	9-22	IFVQKCAQCHTVEK	3	1989.88	N.D.
19	1639.87	1638.93	BSA	437-451	KVPQVSTPTLVEVSR	2	1877.01	1878.19
20	1675.73	1674.79	Lys	116-130	IVSDGNGMNAWVAWR	1	1793.83	1795.01
21	1753.76	1752.83	Lys	64-79	NTDGSTDYGILQINSR	1	1871.87	1873.03
22	1815.83	1814.90	Myo	1-16	GLSDGEWQQVLNVWVK	2	2052.98	N.D.
23	1853.89	1852.95	Myo	80-96	GHHEAELKPLAQSHATK	3	2210.07	2211.28
24	1880.84	1879.91	BSA	508-523	RPCFSALTPDETYPVK	2	2117.99	2119.19
25	2003.72	2002.81	α -Lac	82-98	DDQNPSSNICNISC DK	2	2240.89	2242.09

N.D.: not detected.

shifted by 6 or 9 Da according to the theoretical number of amino groups calculated from the sequence shown in Table 1. From these mass shifts and calculated mass values after derivatization, the sequence of the newly observed signals is matched with the calculated sequence, as shown in Table 1. Thus, owing to the increase in molecular weight and the improved ionization efficiency, this derivatization method was useful for the identification of small peptides such as di- and tripeptides. By using these small peptides 1-6 shown in Fig. 4(g) (*italics*), the sequence coverage was increased from 62% (without derivatization) to 82%.

From the above experiments, the following results were obtained. By using this derivatization method, both the N-terminal amino group and the ϵ -amino group of lysine were completely derivatized. In the case of a C-terminal arginine residue, only a mono-labeled peak (*) was observed. In contrast, in the case of C-terminal lysine residues, only a di-labeled peak (**) was observed. Owing to the increase in molecular weight, the ionization efficiency of small peptides ($m/z < 500$ Da) was significantly improved after derivatization.

To demonstrate the labeling efficiency of amine-reactive reagents for more complexity, a mixture of five proteins (BSA, myoglobin, cytochrome c, α -lactalbumin, and lysozyme) was used. The five-protein mixture was reduced with DTT, alkylated with IAA, and digested with trypsin. Tryptic peptides with or without derivatization were subjected to MALDI mass analysis. The MALDI mass spectra are shown in Fig. 5 and the sequences and mass values of the peptides are summarized in Table 2. Results showed that the labeling efficiency was almost quantitative to the tryptic peptides of protein mixture.

Effect of derivatization on MALDI signal intensities

To examine the effect of the derivatization on MALDI signal intensity, equal amounts of native and peptides derivatized with reagent 1 were mixed and the mixture was subjected to MALDI analysis. Figure 6 shows the MALDI mass spectra of

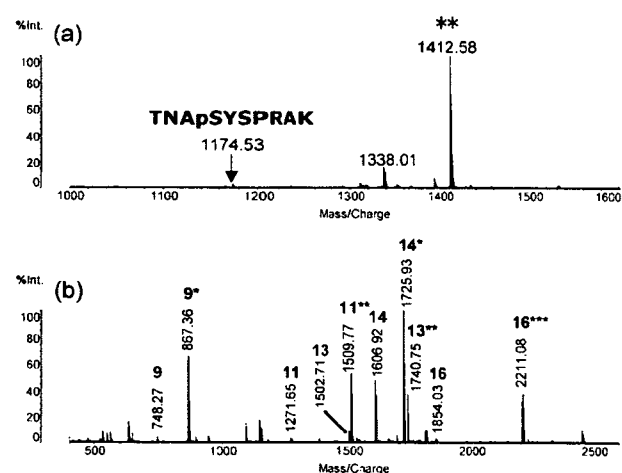


Figure 6. Effect of derivatization on MALDI signal intensity. (a) MALDI mass spectrum of a mixture containing equal amounts of native peptide TNAPSYSPRAK and its peptide derivatized with reagent 1. The signal at m/z 1338.01 is due to the impurity in the sample. (b) MALDI spectrum of a mixture containing equal amounts of native and derivatized tryptic myoglobin. Peaks are marked with entry numbers shown in Table 1. Number of asterisks (*) indicates the number of labels introduced into the peptide by the derivatization.

Table 3. Effect of derivatization on MALDI signal intensities

Entry	Start-End	Sequence	<i>m/z</i>		Signal enhancement (n = 12)
			Native	Labeled	
1	134-139	ALELFR (9 [*] /9)	748.27	867.36	11.7 ± 2.7
2	32-42	LFTGHPETLEK (11 ^{**} /11)	1271.65	1509.77	11.9 ± 2.9
3	119-133	HPGDFGADAQGAMTK (13 ^{**} /13)	1502.71	1740.75	5.0 ± 0.8
4	17-31	VEADIAGHGQEVLR (14 [*] /14)	1606.92	1725.93	1.8 ± 0.4
5	80-96	GHHEAELKPLAQSHATK (16 ^{**} /16)	1854.03	2211.08	8.1 ± 2.8

the mixture containing native and derivatized peptides. The signal-enhancing effect was calculated from the ratio of the signal intensities of the derivatized peptide to those of the native one (Table 3). In the case of the phosphorylated peptide, marked enhancement of ionization efficiency was observed by derivatization with reagent 1 (Fig. 6(a)). In the case of tryptic myoglobin, the derivatization increased detection sensitivity, as shown in Fig. 6(b), and the enhancement factor was approximately 2–11 (Table 3). The signal enhancement of the lysine-containing peptide was greater than that of the arginine-containing peptide. In the case of the arginine-containing peptide, the signal enhancement of the small peptide (9^{*}/9: 11.7) was greater than that of the large peptide (14^{*}/14: 1.8).

Relative quantification by MALDI-TOF MS

To examine the dynamic range of the quantitative analysis, tryptic peptides from myoglobin were used. Two samples

(A and B) containing tryptic peptides at different concentrations were prepared (A: 0.1 µg/µL, and B: 0.5, 0.2, 0.1, and 0.05 µg/µL). Samples A and B were derivatized with isotope-coded light (*d*₀: 1) and heavy reagents (*d*₃: 2), respectively. Equal volumes of solutions derivatized with reagents 1 and 2 were mixed, and the mixture was desalted, concentrated, and subjected to MALDI analysis (2-derivatized, 90–900 fmol). The *d*₃/*d*₀ ratio for doublets spaced by 3 Da was calculated manually using intensities of mono-isotopic peaks (*m/z* 1729/1726). Figures 7(a)–7(d) show the enlarged MALDI-TOF mass spectra of *d*₀- or *d*₃-derivatized tryptic peptides of myoglobin (VEADIAGHGQEVLR). The calculated *d*₃/*d*₀ ratios were 5, 2, 1, and 0.5. The spectra showed that the signal intensities of the doublets spaced by 3 Da changed with the sample ratio. The experimental data are summarized in Figs. 7(e) and 7(f). The correlation coefficient and slope between the expected ratio and the observed ratio for the tryptic peptide VEADIAGHGQEVLR (residues 17–31) were *R*² = 0.9921 and 1.08, respectively, as

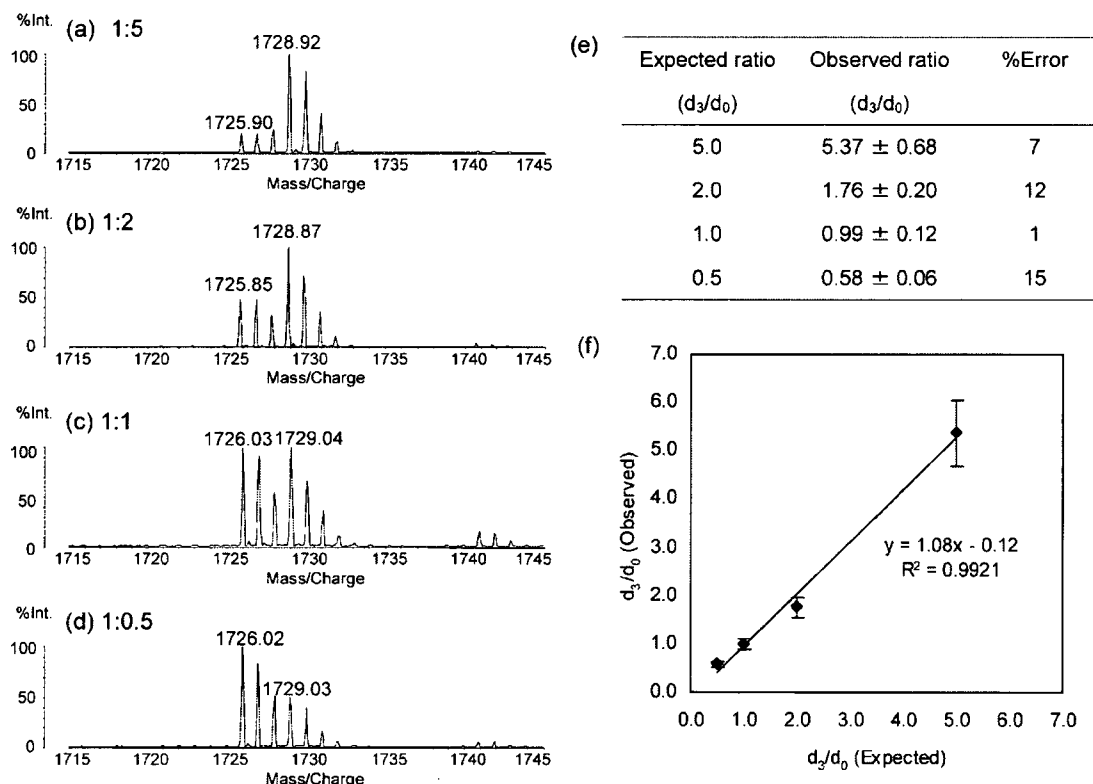


Figure 7. Relative quantification of tryptic digest of myoglobin. Enlarged MALDI mass spectra of peptide VEADIAGHGQEVLR (residues 17–31) derivatized with *d*₀-reagent 1 (*m/z* 1726) and *d*₃-reagent 2 (*m/z* 1729). The ratios of the amounts of *d*₀-derivatized myoglobin and *d*₃-derivatized one were (a) 1:5, (b) 1:2, (c) 1:1, and (d) 1:0.5.

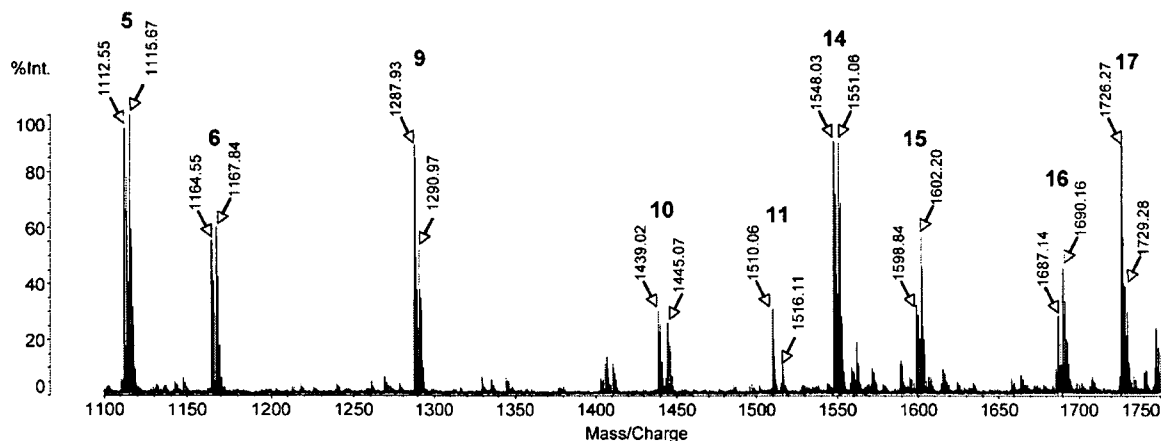


Figure 8. Relative quantification of five-protein mixture. MALDI mass spectrum of a mixture of tryptic digests derivatized with reagents 1 (d_0) and 2 (d_3) is shown. Peaks are marked with entry numbers from Table 2.

shown in Fig. 7(f). These results indicate the potential use of this derivatization method that employs isotope-coded 1 and 2 in the relative quantification of protein by MALDI-TOFMS analysis.

Relative quantification of the five-protein mixture

Two mixtures (A and B) containing the same five proteins at different concentrations were prepared and the applicability of our method was examined. Mixtures A and B consisted of BSA (A: 0.2 $\mu\text{g}/\mu\text{L}$, B: 0.4 $\mu\text{g}/\mu\text{L}$), myoglobin (A: 0.2 $\mu\text{g}/\mu\text{L}$, B: 0.1 $\mu\text{g}/\mu\text{L}$), cytochrome c (A: 0.2 $\mu\text{g}/\mu\text{L}$, B: 0.1 $\mu\text{g}/\mu\text{L}$), lysozyme (A: 0.2 $\mu\text{g}/\mu\text{L}$, B: 0.2 $\mu\text{g}/\mu\text{L}$), and α -lactalbumin (A: 0.2 $\mu\text{g}/\mu\text{L}$, B: 0.2 $\mu\text{g}/\mu\text{L}$), respectively. Samples A and B were reduced, alkylated, digested, and derivatized with reagents 1 and 2 according to the procedure described above. Equal volumes of derivatized samples A and B were mixed and a portion of the mixture was subjected to MALDI analysis. The peptide ratio was calculated from the intensities of the monoisotopic peaks. The MALDI mass spectrum is shown in Fig. 8 and the sequences and ratios of the peptides are summarized in Table 4. Even in the mixtures of five proteins, the protein ratios (d_3/d_0) gave satisfactory values.

CONCLUSIONS

We have demonstrated the applicability of amine-reactive reagents 1 and 2 to the identification and quantification of proteins using the tryptic peptides of model proteins. Our approach involves complete derivatization of both the N-terminal amino groups and the ϵ -amino groups of lysine residues with these reagents. Labeling efficiency was almost quantitative to the tryptic peptides of both a single protein and five-protein mixture. Through the derivatization, the basic moiety of the reagent is incorporated into the amino group, improving the ionization efficiency of the peptide. Isotope-coded reagent 2 (d_3) is useful for the relative quantification of proteins by MS. Moreover, this approach has one advantage for peptide sequence analysis: The peptides generated by tryptic digestion of proteins contain C-terminal arginine or lysine, and these peptides are derivatized with one or two molecules of the reagent, respectively. Comparison of peptides derivatized with reagents 1 (d_0) and 2 (d_3) showed that the derivatization gave mass differences of 3 and 6 Da in arginine- and lysine-containing peptides, respectively. Taken together, the results show that our derivatization method is simple and useful for the identification and relative quantification of proteins.

Table 4. Sequence identification and quantification of proteins in the five-protein mixture

m/z		No. (Table 2)	Protein	Start-End	Sequence	Ratio (d_3/d_0)				
d_0 -Labeled	d_3 -Labeled					Expected	Observed	Average	SD	%Error
1598.84	1602.20	15	BSA	421-433	LGEYGFQNALIVR	2.0	1.9	1.8	0.14	10
1687.14	1690.16	16	BSA	347-359	DAFLGSFLYEYSR	2.0	1.7			
1510.06	1516.11	11	Myo	32-42	LFTGHPETLEK	0.5	0.3	0.4	0.14	20
1726.27	1729.28	17	Myo	17-31	VEADIAGHGQEVLR	0.5	0.5			
1287.93	1290.97	9	Cyt	28-38	TGPNLHGLFGR	0.5	0.5			0
1112.55	1115.67	5	Lys	80-86	WWCNDGR	1.0	1.1	1.1	0.06	10
1164.55	1167.84	6	Lys	135-143	GTDVQAWIR	1.0	1.1			
1548.03	1551.06	14	Lys	52-63	FESNFNTQATNR	1.0	1.0			
1439.02	1445.07	10	α -Lac	118-127	VGINYWLAHK	1.0	0.7			30

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Iron-chelating agents never suppress Fenton reaction but participate in quenching spin-trapped radicals

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Abstract

Hydroxyl radical formation by Fenton reaction in the presence of an iron-chelating agent such as EDTA was traced by two different assay methods; an electron spin resonance (ESR) spin-trapping method with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and high Performance liquid chromatography (HPLC)-fluorescence detection with terephthalic acid (TPA), a fluorescent probe for hydroxyl radicals. From the ESR spin-trapping measurement, it was observed that EDTA seemed to suppress hydroxyl radical formation with the increase of its concentration. On the other hand, hydroxyl radical formation by Fenton reaction was not affected by EDTA monitored by HPLC assay. Similar inconsistent effects of other iron-chelating agents such as nitrilotriacetic acid (NTA), diethylenetriamine penta acetic acid (DTPA), oxalate and citrate were also observed. On the addition of EDTA solution to the reaction mixture 10 min after the Fenton reaction started, when hydroxyl radical formation should have almost ceased but the ESR signal of DMPO-OH radicals could be detected, it was observed that the DMPO-OH^{*} signal disappeared rapidly. With the simultaneous addition of Fe(II) solution and EDTA after the Fenton reaction ceased, the DMPO-OH^{*} signal disappeared more rapidly. The results indicated that these chelating agents should enhance the quenching of [DMPO-OH]^{*} radicals by Fe(II), but they did not suppress Fenton reaction by forming chelates with iron ions.

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Keywords: Electron spin resonance; 5,5-Dimethyl-1-pyrroline *N*-oxide; Hydroxyl radical; Fenton reaction; Ethylenediamine tetraacetic acid

1. Introduction

The spin-trapping method with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been widely accepted as an assay technique to measure the hydroxyl radical scavenging activity of a compound, where the Fenton reaction has been widely used as the source of hydroxyl radicals. In the usual experimental procedure, a compound expected to scavenge hydroxyl radicals is added to the reaction mixture before starting the Fenton reaction. When the electron spin resonance (ESR) signal of the DMPO-OH adducts decreases or disappears, it can be concluded that hydroxyl radicals were scavenged by the additive, or that hydroxyl radical formation was suppressed by the additive only when supporting observations exist which could confirm the for-

mation of a strong chelate with iron ions. Even though such chelate formation should be present, there is no evidence to confirm that hydroxyl radical formation must be suppressed by chelation. As we pointed out previously [1], the signal intensity of DMPO-OH radicals in ESR spectra should diminish in the following cases (Fig. 1):

- (1) OH radical formation is suppressed by the additive,
- (2) the formed OH radical is scavenged by the additive, or
- (3) the formed DMPO-OH radical ([DMPO-OH]^{*}) is quenched by the additive in a short period.

We also demonstrated that Fe(II) ions should quench [DMPO-OH]^{*} radicals. In the same report, we showed that mannitol, which is considered to be a hydroxyl radical scavenger, did scavenge hydroxyl radicals but had little effect on the signal decay of [DMPO-OH]^{*} in ESR, whereas phosphate buffer

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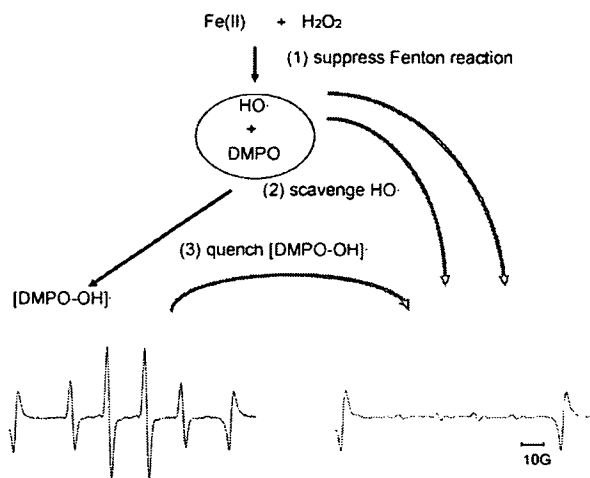


Fig. 1. Three possibilities in DMPO-OH signal decay. (1) The reaction is suppressed, (2) hydroxyl radicals are scavenged, or (3) DMPO-OH radical is quenched by the additive. Chelating agents such as EDTA should participate in this process (3), not in (1). Four peaks in the middle are the signals of DMPO-OH radical ($A_H = 1.50$ mT, $A_N = 1.50$ mT), and the two signals of both ends are those of Mn (external reference).

seemed to scavenge hydroxyl radicals, but this resulted from the enhancement of the DMPO-OH radical quenching activity of Fe(II) ions in the presence of phosphate ions.

The first chelating agent reported to suppress the iron-catalyzed reaction was diethylenetriaminepenta acetic acid (DTPA) [2]. Deferoxamine (DFO) also showed reduced reactivity in $\cdot\text{OH}$ radical formation [3], and is widely used as a “probe” for iron-dependent radical reactions, mostly because its complex with Fe(III) is very difficult to reduce [3,4]; however, ambiguous results have been reported on the effect of EDTA or DTPA on iron-dependent hydroxyl radical-generating systems, varying from stimulation to no effect, to inhibition [5]. Similar conflicting results were observed by Jay [6], who measured the amount of hydroxyl radical formed by Fenton reaction by the deoxyribose degradation method and also ESR measurement with PBN. However, they could not clarify why hydroxyl radical formation was underestimated by ESR measurement, and they showed that the instability of PBN-OH \cdot radical was plausible. Similar variability in the effect of iron-chelating agents was observed by voltammetry [7].

We suspected that these ambiguous effects of iron-chelating agents in hydroxyl radical formation reaction may have resulted from the assay method used. Our previous results indicated that Fe(II) quenched DMPO-OH radicals, and this quenching of [DMPO-OH] \cdot by Fe(II) became much stronger in phosphate buffer where phosphate ions could form a chelate with Fe(II) [1], although we could not demonstrate whether hydroxyl radical formation could be suppressed by chelation. On the other hand, we developed an HPLC assay method for hydroxyl radicals using terephthalic acid as a fluorescent probe [8]. Thus, we tried to confirm the effect of iron-chelating agents in Fenton reaction by measuring two different methods, ESR spin-trapping with DMPO and HPLC.

This report demonstrates that chelating agents with iron ions such as EDTA, NTA and DTPA seem “to suppress the Fenton reaction”; however, hydroxyl radical formation was “not suppressed” by the addition of these chelating agents when observed by the HPLC method, but the addition of these chelating agents enhanced [DMPO-OH] \cdot signal decay greatly by ESR measurement. Thus, it has been clarified that iron-chelating agents enhanced the quenching of DMPO-OH \cdot radical with Fe(II), and the trapped signal diminished in a short period by ESR spin-trapping measurement, which may lead to the mistaken conclusion: “Fenton reaction should be suppressed by iron-chelating agents by forming strong chelation.” This work should be an example that evaluation of the results obtained by a routinely used assay method could clarify an underlying problem.

2. Experiment

2.1. Chemicals

Ammonium iron(II) sulfate (99.999%) was obtained from Aldrich–Sigma Japan (Tokyo, Japan). Hydrogen peroxide (without stabilizer), and other chemicals, such as citric acid, were purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). Most of the chelating agents were obtained from Dojin Chemical Co., (Kyusyu, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan). Sodium terephthalate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and purified by re-precipitation from ethanol and water [8].

The water used in this study was prepared by Milli-Q (Millipore Japan Co. Ltd., Tokyo, Japan), and de-aerated under reduced pressure using an aspirator, then stored under argon by attaching an argon balloon.

2.2. ESR measurement

An electron spin resonance spectrometer, JES-RE1X (JEOL, Tokyo, Japan) and a JEOL flat quartz cell were used. The conditions were: field 336 ± 5 mT width, power 4 mW, field modulation 0.200 mT, time constant 0.1, and amplitude 300. A manganese signal was used for the external standard.

2.3. Solution used for Fenton reaction

Solution A: 5 mmol L⁻¹ DMPO in acetate buffer (117 mmol L⁻¹, pH 5.0).

Solution B: Ammonium ferrous sulfate solution (1 mmol L⁻¹).

Solution C: Hydrogen peroxide solution (0.5 mmol L⁻¹, titrated with permanganate).

Solution D: The additive solution (EDTA, nitrilotriacetic acid (NTA), citrate, oxalate or DTPA at ambient concentration).

Solution E: 5 mmol L⁻¹ sodium terephthalate solution in acetate buffer (117 mmol L⁻¹, pH 5.0).

Table 1
50%-Inhibition concentration (IC₅₀) of DMPO-OH radical formation in Fenton reaction

Compound	IC ₅₀ × 10 ⁻² (mmol L ⁻¹)
DFO	6.1
EDTA	4.8
DTPA	2.1
NTA	1.8
citrate	1.8
oxalate	1.2
Fe(II)	3.0 ^a
Fe(III)	1000.0 ^a
Mannitol	300.0 ^a
Phosphate	10.0 ^a

By ESR measurement, DMPO-OH radical formation was suppressed by the addition of Fe(II) or iron-chelating agents. *Abbreviations:* DFO, deferoxamine; EDTA, ethylenediamine tetraacetic acid; DTPA, diethylenetetramine pentaacetic acid; NTA, nitylotriacetic acid. All data are the average of three measurements.

^a These data are obtained from the previous report [1].

2.4. Measurement of the “suppression” of Fenton reaction

The Fenton reaction was started by mixing 100 μL of solution C to a mixture of 1.7 mL of solution A, 100 μL of solution B and 100 μL of D. The spin-trapped [DMPO-OH][•] signal was measured 2 min after the addition of hydrogen peroxide solution (solution C). In the previous report [1], the signal was recorded 1 min after the addition of H₂O₂; however, signal intensity could be observed more reproducibly after 2 min. The concentrations of the additive changed in the range at which a 50% decrease in the DMPO-OH signal could be observed. The concentration at which 50% scavenging was obtained was expressed as IC₅₀ (50% inhibition concentration for OH radical scavenging) in Table 1.

2.5. Observation of DMPO-OH radical “quenching”

The Fenton reaction was performed by mixing 1.7 mL of solution A, 100 μL of B, and 100 μL of C, which was allowed to stand at room temperature for 10 min. DMPO-OH[•] formation almost ceased after 6 min (96%), and was considered to have completed after 10 min (Fig. 2). Then, 100 μL of a solution of chelating agent, solution D (concentration is IC₅₀, shown in Table 1) was added, and the solution mixed. The [DMPO-OH][•] signal was measured 1 min after the addition of solution D, that is, 11 min after hydrogen peroxide solution (solution C) was added, and successive signal decay was recorded over the next 12 min. The natural logarithm of the signal ratio of [DMPO-OH][•]/Mn was plotted versus time after adding solution D.

2.6. Observation of hydroxyl radical formation by HPLC-fluorescence detection

For HPLC analysis, a JASCO 980 HPLC system (Japan Spectroscopic Co. Ltd., Tokyo) was used, equipped with a Rheodyne sampling valve (20 μL) and a reverse phase column (Develosil

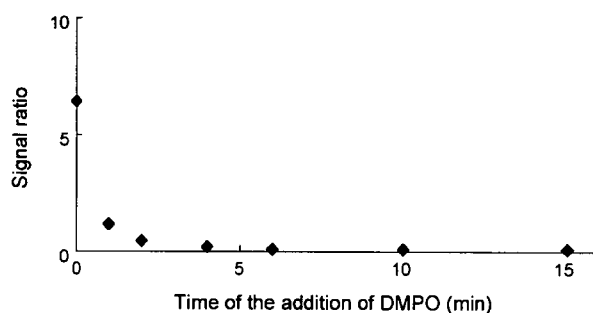


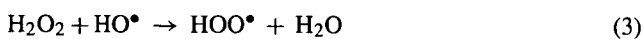
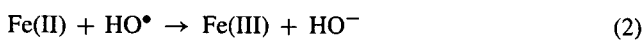
Fig. 2. Fenton reaction almost ceased in ten minutes after the addition of H₂O₂. The datum at 0 min represents the signal ratio of DMPO-OH radical vs. Mn (external standard) 1 min after the Fenton reaction started (by the addition of H₂O₂) in the presence of DMPO. The other data represent the signal ratio of DMPO-OH radical vs. Mn when the reaction had started without DMPO, then DMPO solution was added after the period indicated. Thus, hydroxyl radical formation can be negligible 10 min after the reaction started. Although, the signal of DMPO-OH radical could be detected with an appropriate intensity after 10 min after the reaction started in the presence of DMPO.

C30-UG5, 4.6 mm i.d. × 250 mm, Nomura Chemical Co., Seto, Aichi, Japan; this column could be used without any organic additive in high reproducibility in retention profile [9]). It was eluted with 200 mmol L⁻¹ potassium hydrogen phosphate containing 2% of KCl (pH 4.37) and monitored with a UV detector 750-UV (290 nm, Japan Spectroscopic Co. Ltd., Tokyo) and a fluorescence detector, FP1520-S (Japan Spectroscopic Co. Ltd., Tokyo) in series. The excitation and emission wavelengths were: Ex, 309 nm and Em, 412 nm, respectively.

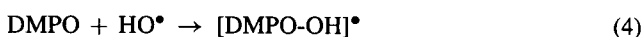
Fenton reaction was performed as described in the Section 2.4, except for the use of a solution of sodium terephthalate, solution E, instead of solution A (DMPO solution).

3. Results and discussion

The initial reaction of the Fenton reaction is described as Eq. (1), accompanied by reactions (2) and (3) [10,11]. Yamazaki and Piette [12] found that the stoichiometry of oxidant formation in the reaction between Fe(II) and H₂O₂ often shows marked deviation from the expected value of 1:1, in the observation with ESR. Sharpe and Cooper also reported that the Fenton reaction became unstable when the ratio of Fe(II) to H₂O₂ exceeded 1.0 [13]; however, under our reaction conditions, we used Fe(II):H₂O₂ = 2:1, because the reaction reached a plateau at this molar ratio by observation of the ESR method.



In the presence of DMPO, hydroxyl radicals were trapped by DMPO (Eq. (4)).



The signal of [DMPO-OH][•] could be observed for 30 min after the addition of hydrogen peroxide under the conditions described above; however, in the presence of EDTA, NTA,

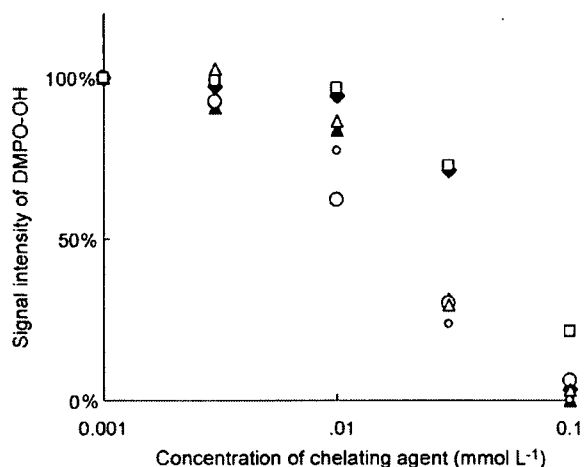


Fig. 3. Iron-chelating agents inhibited DMPO-OH formation in Fenton reaction in the presence of DMPO (citrate (Δ), oxalate (\circ), NTA (\diamond), EDTA (\blacklozenge), DFO (\square), and DTPA (\blacktriangle)). The 50%-inhibition concentration (IC_{50}) of each chelating agent was obtained, and was shown in Table 1.

DTPA, citrate or oxalate at the beginning of the Fenton reaction, the signal of DMPO-OH diminished depending on the concentration of these additives (Fig. 3). Thus, 50% suppression concentration (IC_{50}) of each compound was determined (Table 1), where Fe(II) and Fe(III) were also studied for their scavenging effects. It seems that Fe(II) and these chelating agents are efficient hydroxyl radical “scavengers” or “suppressors of the Fenton reaction”.

Iron-chelating agents, such as deferoxamine (DFO), are known to prevent Fenton reaction by complex formation with Fe(II) ion [3]. This was deduced from observation of ESR spin-trapping measurements with DMPO and lipid oxidation measured by red pigment formation with *thiobarbituric acid* (TBA); however, EDTA solely inhibited the TBA formation reaction [14]. Also, Aitken et al. [15] reported that EDTA suppressed malondialdehyde formation in vivo, but hydroxyl radical formation was enhanced by the addition of EDTA. Thus, the “chelating effect” must be checked by a different analytical method.

Under the conditions for the Fenton reaction described here, the reaction almost ceased about 10 min after the addition of hydrogen peroxide solution (Fig. 4). When the Fenton reaction started in the presence of DMPO, the hydroxyl radical formed should be trapped by DMPO, and its formation should almost cease by 10 min, but the DMPO-OH signal could be detected for more than 20 min thereafter. If the DMPO-OH \cdot signal disappeared by the addition of a compound to the reaction mixture, we could suspect that the additive should decompose [DMPO-OH] \cdot . Thus, the signal decay of [DMPO-OH] \cdot was monitored after the addition of a chelating agent 10 min after the Fenton reaction started. It was found that the [DMPO-OH] \cdot signal diminished in a short period after the addition of these chelating agents, whereas the addition of mannitol and dimethyl sulfoxide, which are hydroxyl radical scavengers, or acetate buffer (pH 5.0, as control), did not affect signal intensity (Fig. 5). When these chelating agents in a fresh Fe(II) solution were added, the

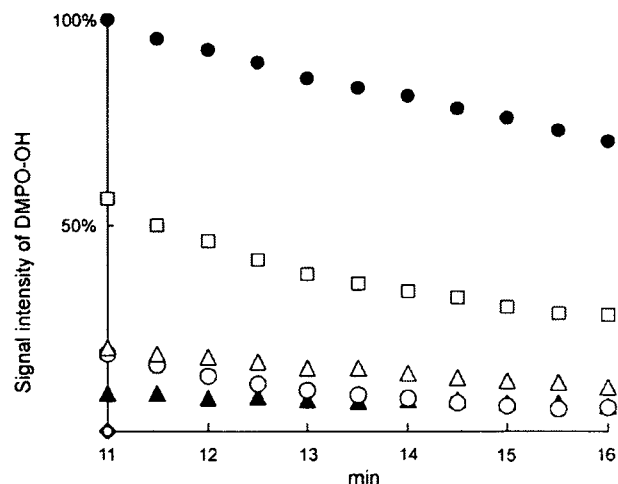


Fig. 4. ESR signal of DMPO-OH radical decreased rapidly by the addition of chelating agents (DFO (\square), citric acid (Δ), oxalic acid (\circ), DTPA (\blacktriangle)) after the Fenton reaction finished (10 min after the reaction started), but changed little by the addition of water (\bullet). With NTA (\circ) and EDTA (\blacklozenge), the DMPO-OH signal disappeared completely (0% at 11 min). These results indicate that these chelating agents should not suppress the Fenton reaction, but may diminish DMPO-OH radical.

DMPO-OH \cdot signal disappeared quite rapidly (Fig. 6). Thus, it was suspected that [DMPO-OH] \cdot is decomposed to a diamagnetic specimen by Fe(II) in the reaction mixture. We speculate that these chelating agents enhance the quenching of [DMPO-OH] \cdot with Fe(II).

In the compounds listed in Table 1, EDTA, NTA and DTPA were subjected to the HPLC assay of hydroxyl radical formation. As shown in Fig. 4, hydroxyl radical formation detected by the hydroxylation reaction of terephthalic acid [8] showed that these chelating agents had little effect on hydroxyl radical formation and, with DTPA, enhancement of hydroxyl radical formation was observed at a higher concentration. It has been reported that

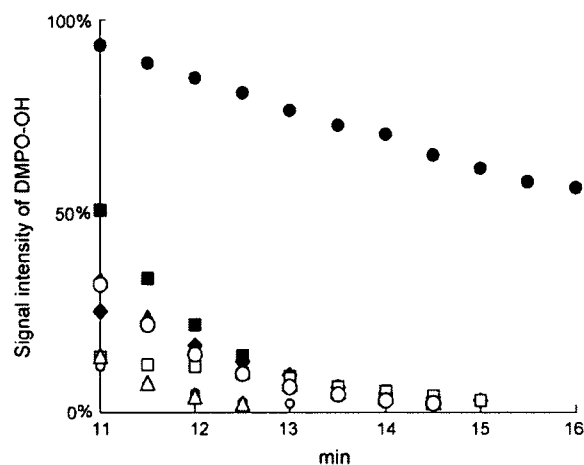


Fig. 5. The signal of DMPO-OH radical quenched by the addition of Fe(II) (\blacksquare). The DMPO-OH radical quenching of Fe(II) was enhanced by the simultaneous addition of Fe(II) and chelating agents (citrate (Δ), oxalate (\circ), NTA (\diamond), EDTA (\blacklozenge), DFO (\square), and DTPA (\blacktriangle)), although it was not affected by the addition of water (\bullet).

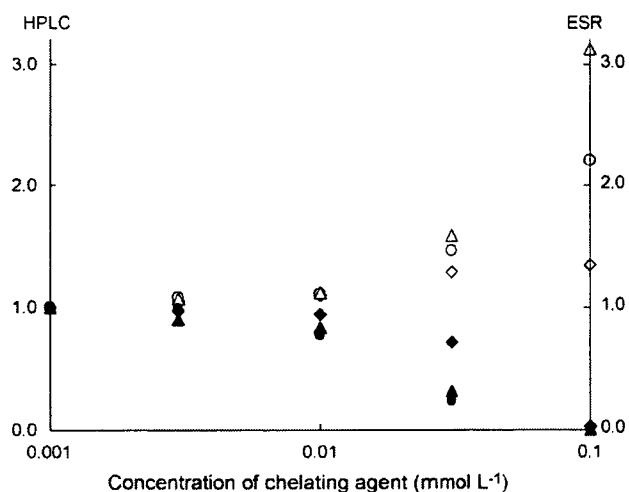


Fig. 6. Measurements of hydroxyl radical formation in Fenton reaction by two different methods. Formation ratios of hydroxyl radical indicators are measured relative to that in the presence of iron-chelating agent of $0.001 \text{ mmol L}^{-1}$; formation of DMPO-OH by ESR spin-trapping method with DMPO (DTPA (▲), EDTA (◆), NTA (●)), and formation of hydroxyl terephthalate detected by HPLC with terephthalic acid (DTPA (△), EDTA (◇), NTA (○)). Fenton reaction was not suppressed by these iron-chelating agents measured by HPLC.

DMPO forms [DMPO-OH]^{*} in Fe(III) solution [16], or Fe(III) in the presence of chelating agents such as DTPA or DFO [17]. Accordingly, the increase of hydroxylated terephthalate may be considered as the contribution of Fe(III)-aided hydroxyl radical formation in the presence of these strong iron-chelating agents.

It must be pointed out that hydroxyl radical scavenging measurements by the ESR-spin trapping method with DMPO may lead to the mistaken conclusion that “the chelating agents with iron ions should suppress Fenton reaction by chelate formation,” when the Fenton reaction is used as the hydroxyl radical source with an iron-chelating agent.

4. Conclusions

In conclusion, it was demonstrated that hydroxyl radical formation in the Fenton reaction increased in the presence of chelating agents with iron ions such as EDTA, whereas the

ESR signal of DMPO-OH radicals disappeared rapidly with Fe(II) ions, and this quenching effect was enhanced by chelating agents. When we measure changes in the amount of hydroxyl radical formed in the Fenton reaction, it must be measured by several different assay methods, such as ESR, HPLC, TBA reaction and so on, and we must be careful in our assessment of the results obtained.

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