glutamine (Gln) at the C-terminus, whereby it is impossible to discriminate between the corresponding amide forms of aspartic acid (Asp-NH_2) and glutamic acid (Glu-NH_2), unless an additional chemical or enzymatic approach is implemented. In view of such a technical difficulty in detection, it might not be too suspicious to speculate that at least a small proportion of mature proteins could bear C-terminal modification without having been identified as yet.

As is the general case with PTMs, a protein presumed to have a particular type of C-terminal modification can be isolated or enriched by a specifically designed scheme based on either affinity chromatography or enzyme treatment. The RIA is widely used for the detection and quantification of C-terminal amidated peptides, which amount to more than half of the known neural and endocrine peptides [7]. Immunoprecipitation targeting of bioactive peptides with particular amino acid sequences including the C-terminal amide group has made it possible to identify two novel amidated peptides [8]. However, a higher specificity of the method for analyzing the C-terminal modification imposes a narrower range of target proteins, thus severely limiting the applicability of the method. Probably, the most reliable method for distinguishing between free and modified carboxyl groups at the C-terminus of a protein is by a chemical or enzymatic approach.

We suggest here a chemical method for detecting PTMs at the C-termini of peptides and proteins, utilizing a technique for the isolation of the C-terminal peptides [9] in combination with a reaction for selectively derivatizing the C-terminal carboxyl group according to oxazolone chemistry [10, 11]. The scheme for C-terminal derivatization is specifically designed to convert the free C-terminal carboxyl group into methylamide ($CONHCH_3$), increasing the mass of the peptide by 13 Da, while peptides possessing the amide ($CONH_2$) group remain unchanged (Fig. 1). Because an oxazolone is formed solely at the C-terminus, the existence of free carboxyl side chains of Asp and Glu does not affect the performance of the present method. The relatively low yield of the reaction conducted in the manner of the original protocol [11] is improved to the extent that the methylamidated peptide can be detected as a MALDI peak appearing at a higher mass value by 13 Da than that of the original peptide, with more than comparable intensity in the mass spectrum. This should make it easier to distinguish between peptides with the free C-terminal carboxyl group and those with the post-translationally modified C-terminal carboxyl group, based on the presence or absence of an additional MALDI peak.

In this report, we present a new approach for detecting C-terminal amides in proteins and peptides by mass spectrometry.

2 Materials and methods

BSA, acetic anhydride (Ac_2O), 40% methylamine solution (w/w, aqueous), and iodoacetamide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human adrenomedullin, human calcitonin, VYIHPF, and WMDF-NH_2 were purchased from the Peptide Institute (Osaka, Japan). Tris-(2-carboxyethyl)phosphine hydrochloride and succinimidyloxycarbonylmethyl Tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-Ac-OSu) were obtained from Fluka (Switzerland). Lysyl endopeptidase (LysC), ACN, 2-propanol, formic acid, pentafluorophenol (Pfp-OH), and TFA were purchased from Wako Pure Chemical Industries (Osaka, Japan). CHCA (high-purity mass-spectrometric grade) was obtained from Shimadzu GLC (Tokyo, Japan). TNVGSEAF-NH_2 and PVTL-OCH_3 were obtained from Bachem AG (Switzerland). HPTFD-NH_2, SFLLRN-OH,

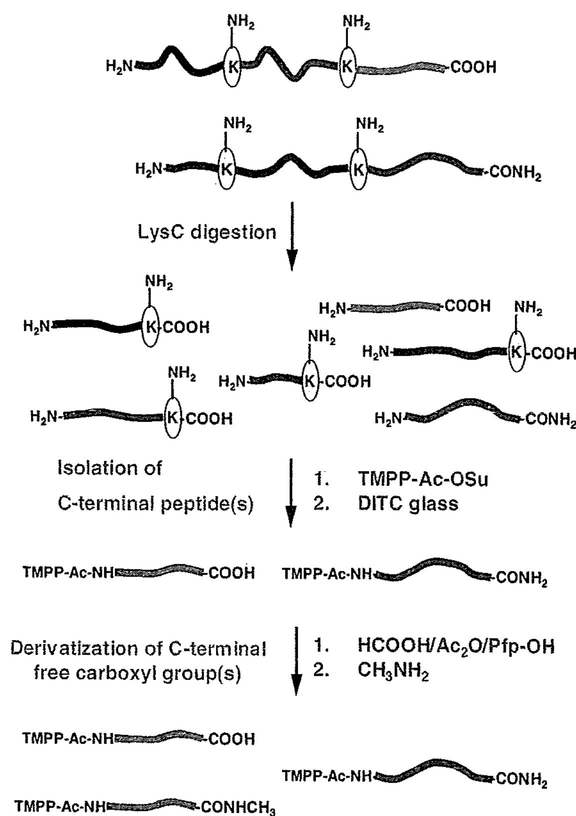

Figure 1. Scheme depicting the isolation of C-terminal peptides and subsequent procedure to distinguish between peptides with C-terminal amide ($CONH_2$) as an example of PTM and those with the ordinary free carboxyl group (COOH). C-Terminal peptides of proteins are prepared, as reported previously [9]. At the last step of the procedure, C-terminally modified peptides are inert to the reaction to derivatize the C-terminal-free carboxyl group, which is selectively converted to the methylamide according to the oxazolone chemistry [11]. The difference in the chemical form of C-termini can be discerned by the presence of or absence of mass shift resulting from the derivatization. Owing to the general incompleteness of the derivatization of the free C-terminal carboxyl group with methylamine, the resulting preparation exhibits a doublet peaks in mass spectrum separated by 13 Da corresponding to the difference in mass of COOH and $CONHCH_3$ groups.

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TTSFAE-NH_2, and ALEGSLQ-OH were purchased from Operon Biotechnologies, K.K. (Tokyo, Japan). The *p*-phenylenediisothiocyanate glass used in this study was prepared in house according to the method of Wachter *et al.* [12], using aminopropyl glass (average pore size 170 Å, 200–400 mesh, amine content: 162 μmol/g) obtained from Sigma. Methanediphosphonic acid (MDPNA) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Water used in all the experiments was purified using a MilliQ water purification system. All other chemicals were of analytical reagent grade and were used without further purification.

2.1 C-Terminal amidation

C-Terminal peptides (20 pmol) were prepared from proteins or peptides, as reported previously [9]. The isolated TMPP-Ac-modified C-terminal peptides were purified with ZipTip

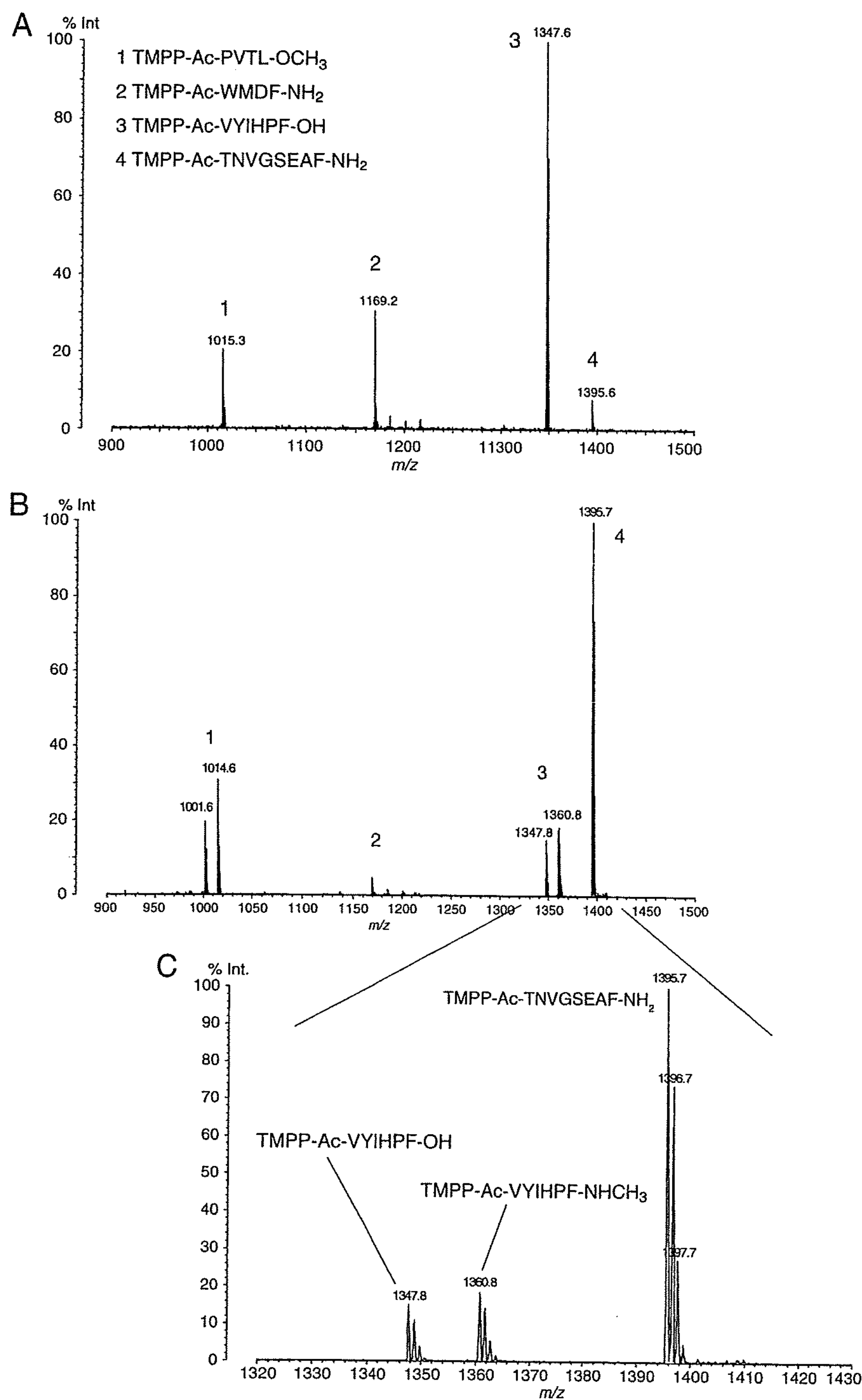

Figure 2. MALDI-TOF mass spectra of a four-peptide mixture (PVTL-OCH_3, WMDF-NH_2, VYIHPF-OH, and TNVGSEAF-NH_2): (A) TMPP-Ac modification; (B) methylamidation; and (C) enlarged view of peptides 3 and 4 after methylamidation: peptide 3 (TMPP-Ac-VYIHPF-OH) having free C-terminal carboxyl group is converted to its methylamide (TMPP-Ac-VYIHPF-$NHCH_3$).

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and dried using a vacuum concentrator. Next, 12 μL of HCOOH–Ac_2O–Pfp-OH mixture (1:1:1 in volume) was added to the resulting dried peptides, and the mixture was incubated for 20 min at 60°C, followed by evaporation to dryness using a vacuum concentrator. The dried material was dissolved with 14 μL of a 5:2-mixture of toluene and Pfp-OH, and dried again. Ten microliter of 40% (w/w, aqueous) CH_3NH_2-ACN (1:9) was then added to the resulting peptides; the mixture was subsequently sonicated for 10 min and left standing for 20 min. For mass analysis, an aliquot (1 μL) was acidified with aqueous TFA solution and purified with ZipTip.

2.2 MALDI-TOF MS

MALDI mass spectra were recorded on AXIMA CFR-plus or AXIMA TOF^2 (SHIMADZU/KRATOS, Manchester, UK) reflectron time-of-flight mass spectrometers equipped with a nitrogen laser (337 nm, 3 ns pulse width). All measurements were performed in the positive-ion reflectron mode. The ion acceleration voltage was set to 20 kV, and the reflectron detector was operated at 24 kV. The flight path in the reflectron mode is 240 cm for both instruments. For the MS/MS experiments, CID was carried out using helium at a pressure of ca. 5×10^{-6} mbar in the collision cell.

The matrix used in this experiment was CHCA, which was dissolved to saturation in 50% aqueous ACN containing 0.05% TFA. We used MDPNA, which has been proven useful for MALDI analysis of salt-containing samples, as a matrix additive [13]. MDPNA was used as 1–2% aqueous solution. An aliquot (0.4 μL) of the sample solution was mixed with an equivalent volume of matrix solution and matrix additive solution on the MALDI target plate and analyzed after drying.

The *m/z* values in the spectra were externally calibrated with angiotensin II (human) and ACTH fragment 18–39 (human) using CHCA as a matrix.

3 Results and discussion

3.1 Amidation of model peptides

C-Terminal-specific amidation reaction and its side reaction(s) were investigated using peptides VYIHPF-OH, TNVGSEAF-NH_2, WMDF-NH_2, and PVTL-OCH_3. A mixture of four peptides (20 pmol each) was processed as follows: (i) N^{α}-modification with TMPP-Ac-OSu (Fig. 2A), (ii) selective activation of C-terminal carboxyl group to the active ester through oxazolone, and (iii) amidation with methylamine (Fig. 2B). In principle, this process should cause peptides with the free C-terminal carboxyl group to increase in mass by 13 Da, while causing no change in mass for those modified at the C-termini. Owing to the incomplete C-terminal amidation, a new peak of TMPP-Ac-VYIHPF-$NHCH_3$ arose at the higher mass value by 13 Da, along with the original one for TMPP-Ac-VYIHPF-OH (Fig. 2C). The formation of TMPP-Ac-VYIHPF-$NHCH_3$ was confirmed by *de novo* sequencing of the resulting peak by MS/MS in CID mode (Fig. 3). As expected, the C-terminally amidated peptide (TMPP-Ac-TNVGSEAF-NH_2) remained intact through C-terminal activation and methylamidation (Fig. 2C).

In the mass spectrum of peptide TMPP-Ac-WMDF-NH_2 containing aspartic acid along with the C-terminal amide and having been submitted to the reaction, no peak indicating amidation of the side-chain carboxyl group of aspartic acid appeared. As is the case with aspartic acid, the side-chain carboxyl group of glutamic acid has also proved to be virtually inert to methylamidation in this protocol (TMPP-Ac-TNVGSEAF-NH_2 in Fig. 2C). Thus, the C-terminal and side-chain carboxyl groups have been distinguished successfully.

A few marginal peaks of by-products or artifacts were detected, while this peptide has no C-terminal carboxyl group to be methylamidated (Fig. 2B). Observation of peaks at *m/z* 1185.5 (+16 Da) and *m/z* 1201.6 (+32 Da) is consistent with the possible oxidation of methionine to its sulfoxide and sulfone forms. Although tryptophan is also susceptible to oxidation, the appearance of peaks at *m/z* 1213.6 (+16+28 Da) and *m/z* 1227.5 (+16 × 3 Da) suggests that formylation (+28) might have occurred in preference to oxidation (Fig. 4B). The latter peak could correspond to the peptide incorporating two oxygen atoms in methionine and one in tryptophan. These reactions are predictable [11] and clearly distinguishable from the desired one, allowing for the shift of mass values. Under the present reaction conditions, the α- and ε-amino groups are also formylated. However, the TMPP-Ac derivative of isolated C-terminal peptide is devoid of these amino groups, thus causing no problem [9].

In the peptide TMPP-Ac-PVTL-OCH_3, the C-terminal methyl ester was found to undergo hydrolysis and aminolysis simultaneously to give TMPP-Ac-PVTL-OH with the signal at *m/z* 1001.6 and TMPP-Ac-PVTL-$NHCH_3$ with that at *m/z* 1014.6 as shown in Fig. 4A. However, it is possible that the methyl-amidated peptide occurred *via* a sequential reaction of hydrolysis and amidation is not excluded.

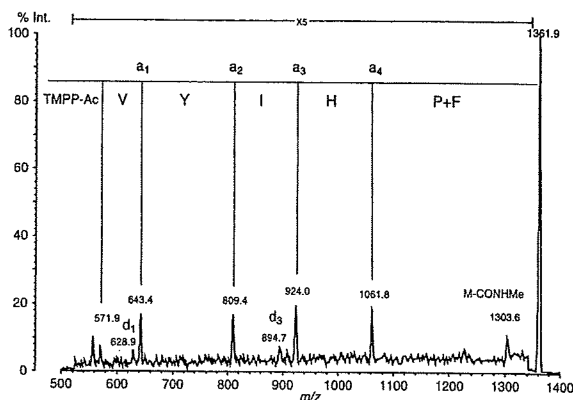

Figure 3. MALDI-CID spectrum of peptide 3 methylamide derivative (TMPP-Ac-VYIHPF-$NHCH_3$).

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formylation of amino and hydroxyl groups. If necessary, the mass of methylamine may be altered by isotopically labeling any atom(s) to further accentuate the distinction between the amide and free carboxyl forms of the C-termini, of which masses intrinsically differ as little as 1 Da. With such versatility in the choice of reagent with varied mass values, the present method should be applicable to *de novo* amino acid sequencing of any kind of PTM to occur at the C-termini. Although the additional task of C-terminal derivatization may reduce the throughput of the original protocol [9], this method has a practical feasibility for identifying C-terminal PTMs with high fidelity, which has usually been very difficult to attain.

4 Concluding remarks

The development of a chemical method for detecting C-terminal PTMs of peptides and proteins is described. The method is based on oxazolone chemistry, which enables the specific derivatization of the C-terminal-free carboxyl group to, for example, methylamide by reaction with methylamine, while leaving the side-chain carboxyl groups unchanged. One of the most promising applications of this method is to detect and characterize C-terminally amidated peptides and proteins because the small mass difference of 1 Da between masses of the free carboxyl group and its amide is expanded to 14 Da due to the conversion of the carboxyl group to the corresponding methylamide. The C-terminal amide structures of adrenomedullin and calcitonin are unambiguously discriminated from the free C-terminus of BSA. The efficacy of this method is such that it can determine even the isobaric variations in C-terminal structures corresponding to 132 Da (Asp-NH_2 and Asn-OH) and 146 Da (Glu-NH_2 and Gln-OH). These features suggest that the present method has a wider applicability to determine the existence or nonexistence of PTMs at the C-termini of peptides and proteins.

The authors have declared no conflict of interest.

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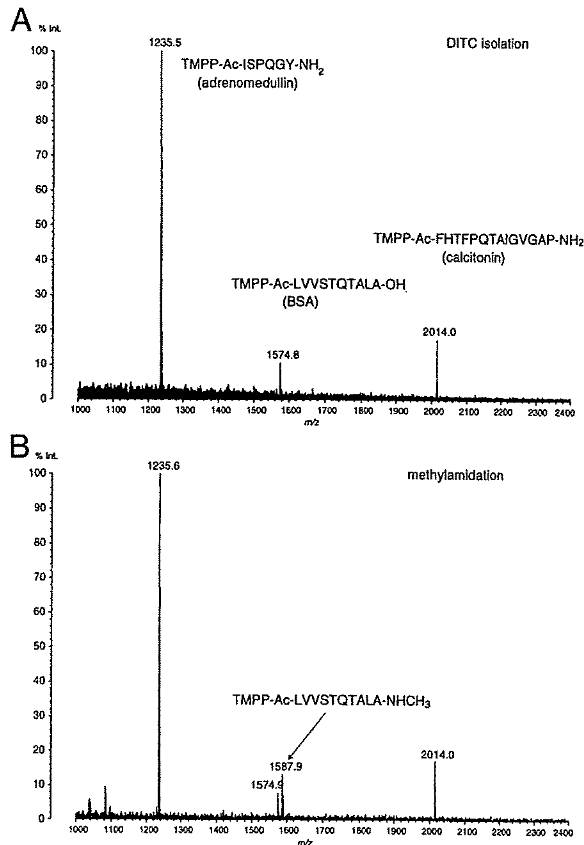

Figure 8. MALDI-TOF mass spectra of protein/peptide mixture: (A) isolated three C-terminal peptides from BSA (*m/z* 1574.8), adrenomedullin (*m/z* 1235.5), and calcitonin (*m/z* 2014.0). (B) Methylamidation converts the C-terminal peptide from BSA to its methylamide form, whereas the other two peptides are intact.

any change in mass, while C-terminal peptide from BSA underwent methylamidation properly to give TMPP-Ac-LVVSTQTALA-$NHCH_3$, the molecular mass of which is higher than that of the C-terminally free precursor by 13 Da. Figure 8B depicts the MALDI mass spectrum of the mixture of TMPP-Ac-ISPQGY-NH_2 from adrenomedullin and TMPP-Ac-FHTFPQTAIGVGAP-NH_2 from calcitonin thus processed. The identity of TMPP-Ac-LVVSTQTALA-$NHCH_3$ derived from BSA was confirmed by tandem mass spectrometry (Fig. 9). Each of these isolated peptides contains a few hydroxyl groups of serine, threonine, or tyrosine, where formylation can occur concomitantly with the activation of the C-terminus to oxazolone in the mixture of formic acid and acetic anhydride. However, no formylation was detected in these peptides, probably because the hydroxyl groups could be restored by aminolysis of the formate during the reaction with methylamine [11].

3.4 Reagents and improvement of protocol for future development

We chose to employ methylamine for amidation of the C-terminal carboxyl group, because it is the simplest alkylamine that is least likely to suffer from any steric hindrance to the reaction with the activated C-terminal carboxyl group. In addition, it can be used in large excess with the peptides to be amidated, due to its high solubility in any solvent. This is particularly important to suppress hydrolysis, which could lower the yield of methylamidation; there is the risk of reporting false-positive results in the mass spectra when the yield of the reaction is very poor or negligibly low. Methylamidation of a carboxylic acid is accompanied by an increase in the molecular mass by 13 Da, making it easier to discriminate from the mass shift caused by other side reactions such as

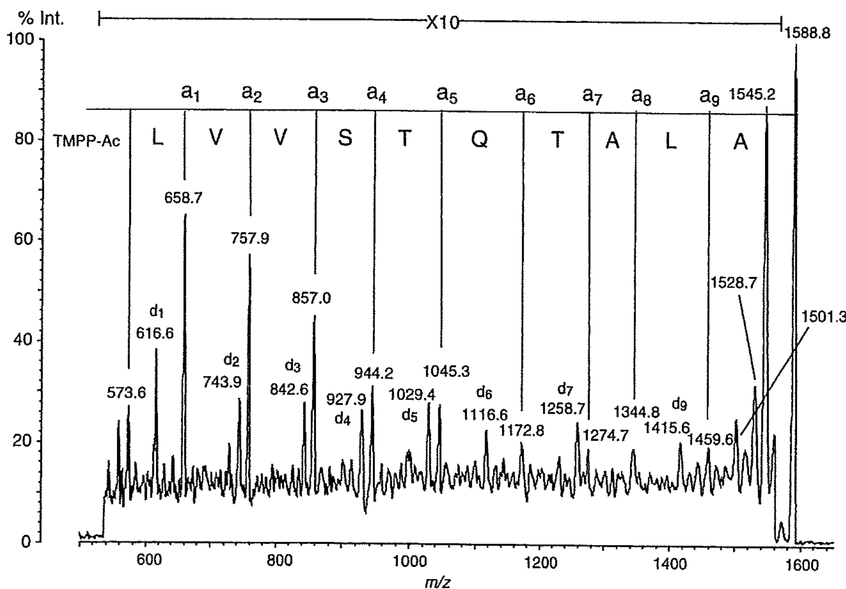

Figure 9. MALDI-CID spectrum of a methylamidated C-terminal peptide from BSA (TMPP-Ac-LVVSTQTALA-$NHCH_3$).

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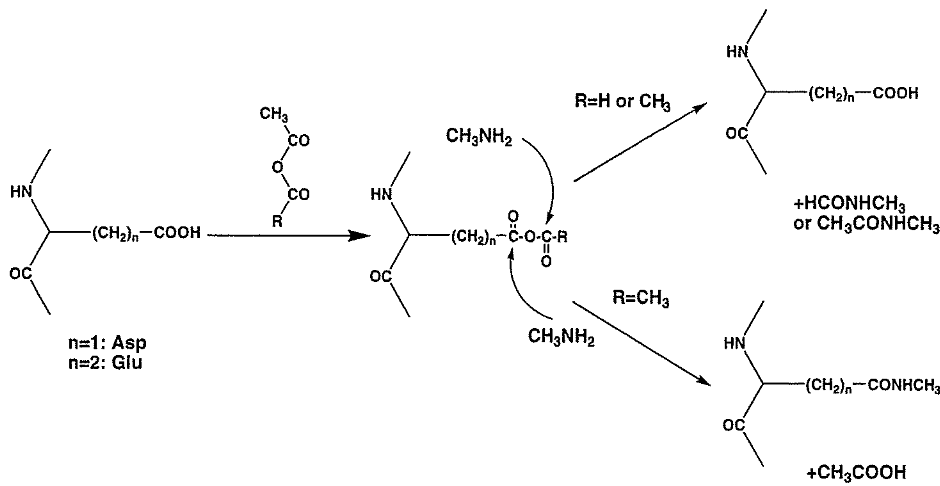

Figure 6. Possible involvement of acetic anhydride ($R = CH_3$) in the false activation of the side-chain carboxyl group to the reaction with methylamine. The mixed anhydride with acetic acid allows the reaction to proceed in both directions of leaving the carboxyl group free and changing it to the amide form.

twice (at the initial and final steps of C-terminal activation) so that the undesirable hydrolysis of oxazolone or active ester could be suppressed. Although the yield of amidation appeared to become higher by increasing the proportion of Ac_2O in the reagent (*e.g.* HCOOH–Ac_2O–Pfp-OH = 1:1:1, 1:3:1, 1:5:1, *etc.*), the specificity of methylamidation to the C-terminal carboxyl group was adversely affected (data not shown). This was possible because the side chains of aspartic acid and glutamic acid can be activated due to the formation of mixed anhydride with acetic acid, allowing methylamine to react with the activated side-chain carboxyl group as well as the acetyl group of the mixed anhydride (Fig. 6). In the proper condition of 1:1 mixture of formic acid and acetic anhydride, acetic anhydride is almost completely converted to formic acetic anhydride, which then reacts with any carboxyl group to form the mixed anhydride with formic acid in preference to that with acetic acid. It is far less likely that the resulting mixed anhydride with formic acid leads the side-chain carboxyl group to be amidated. Therefore, the modest yield in the range of 60–70% is sufficient to distinguish between the free and modified C-terminal carboxyl groups, according to the appearance of MALDI mass peak(s) in a single spectrum. Given the recognition of the peak of interest, the MS/MS C-terminal amino acid sequencing of the peak would follow for the identification of the peptide and the characterization of C-terminal modification.

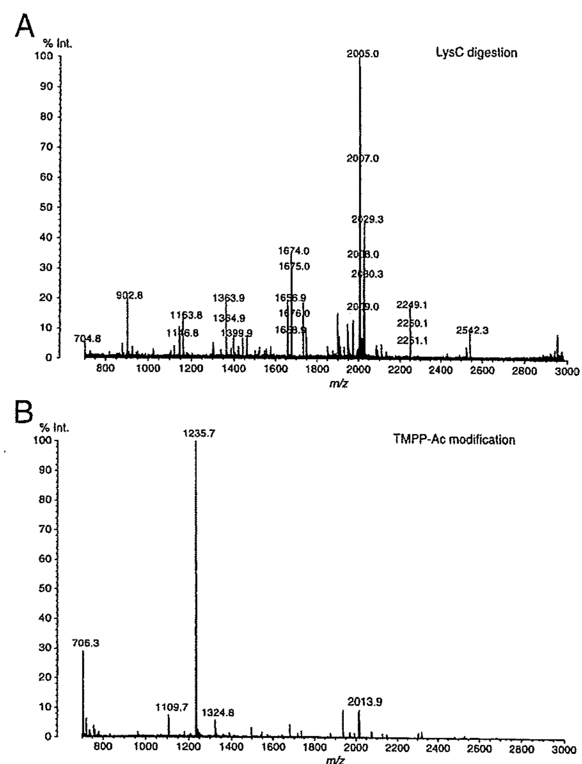

Figure 7. MALDI-TOF mass spectra of the protein/peptide mixture containing BSA, human adrenomedullin, and human calcitonin: (A) LysC digest and (B) TMPP-Ac modification.

3.3 Amidation of the model protein and large peptides

To test the applicability of the method to proteins or large peptides, we tried to isolate and characterize the C-terminal peptides by using a mixture of BSA, human adrenomedullin, and human calcitonin (10 pmol each). Of these samples, adrenomedullin (52 amino acid residues; 6028 Da) has Tyr-NH_2, and calcitonin (32 amino acid residues; 3417 Da) has Pro-NH_2 at the C-termini, distinguished from BSA having the free C-terminal carboxyl group. The mixture was subjected to LysC digestion, followed by N-terminal modification with TMPP-Ac-OSu and isolation of C-terminal peptides by the treatment with *p*-phenylenediisothiocyanate glass, according to the standard protocol [9]. MALDI mass spectra recorded at each step of the procedure are indicated in Fig. 7. Owing to the absence of free carboxyl groups at their C-termini, neither the C-terminal peptide derived from adrenomedullin nor that derived from calcitonin indicated

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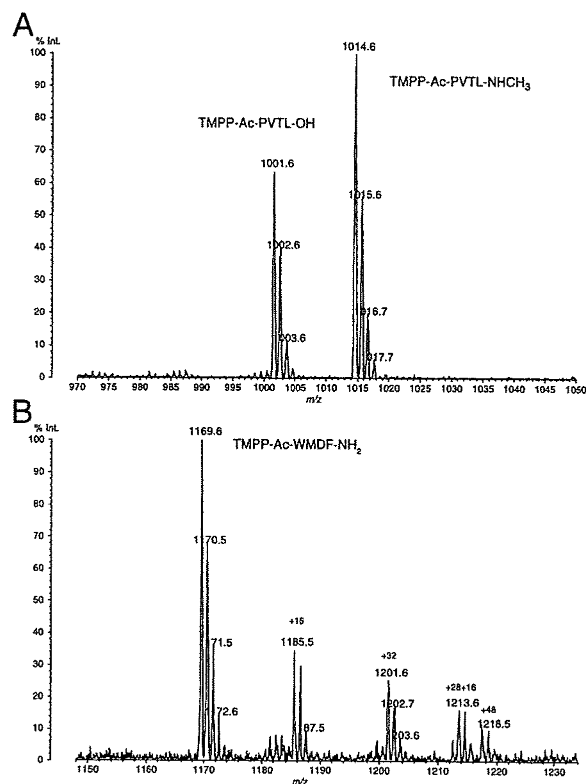

Figure 4. Peptides 1 and 2 after methylamidation: enlarged spectra from Fig. 2B. (A) peptide 1 (TMPP-Ac-PVTL-OCH_3) and (B) peptide 2 (TMPP-Ac-WMDF-NH_2).

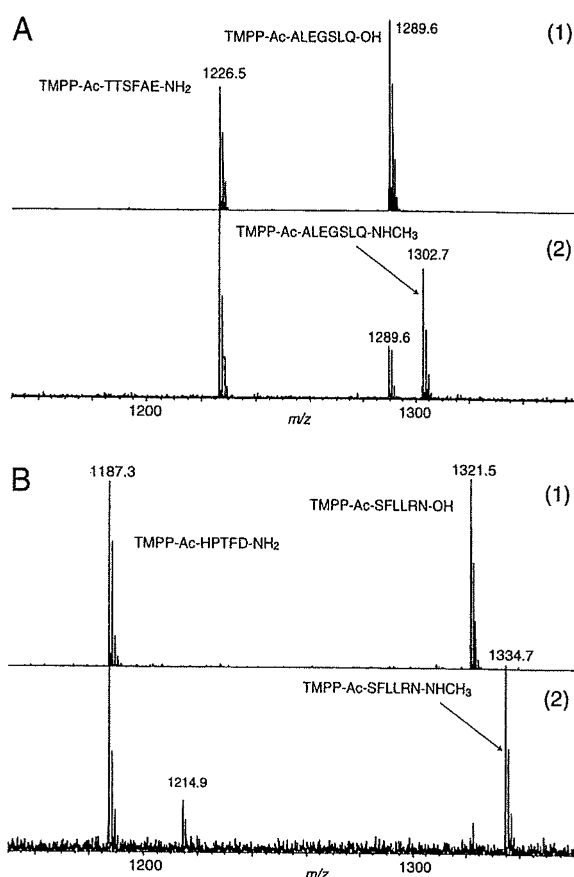

Figure 5. Methylamidation of two pairs of peptides: (A) TMPP-Ac-TTSFAE-NH_2, TMPP-Ac-ALEGSLQ-OH and (B) TMPP-Ac-HPTFD-NH_2, TMPP-Ac-SFLLRN-OH. Peptide having Asn (N) or Gln (Q) at the C-terminus is converted to its methylamide (+13 Da), whereas peptide incorporating the amide form of aspartic acid (D) or glutamic acid (E) is intact.

Regardless of the course by which the ester has been converted to the amide, any kind of ester could be analyzed in this manner by assuming the same reactions and results.

3.2 Discrimination between the C-terminal and side-chain carboxyl groups

As we presumed in the Introduction, in the unfortunate situation in which the C-terminus of a peptide is Asn-OH/Gln-OH or amidated Asp-NH_2/Glu-NH_2, it is impossible to discriminate between these isobaric residues by mass spectrometry alone. However, the present method can achieve the requisite discrimination by causing a mass shift by +13 Da for peptides having free Asn or Gln at the C-terminus through methylamidation, but no change for those with C-terminal amide. We further investigated the validity of this approach using two pairs of model peptides: HPTFD-NH_2/SFLLRN-OH and TTSFAE-NH_2/ALEGSLQ-OH. As expected, the peptide derivatives TMPP-Ac-HPTFD-NH_2 and TMPP-Ac-TTSFAE-NH_2 containing the amide form of aspartic and glutamic acids at their C-termini remain intact after methylamidation, whereas C-terminal-free TMPP-Ac-ALEGSLQ-OH and TMPP-Ac-SFLLRN-OH are converted to the corresponding methylamides with the increment of mass value by 13 Da (Figs. 5A and B). An additional peak accompanied to that of TMPP-Ac-HPTFD-NH_2 (Fig. 5B-2) seems to arise from the formylation (+28 Da) of the histidine or threonine residue during methylamidation. Nevertheless, no confusion due to the side-chain formylation might be imposed on the discrimination of peptides entailing this isobaric ambiguity.

The efficiency of our method for the detection of C-terminal amide and the discrimination between the C-terminal and side-chain carboxyl groups depends on the yield of methylamidation, which was usually incomplete so that the yield was 60–90%, as roughly estimated from the signal intensities of several pairs of peaks separated by 13 Da each (Figs. 2B, 5A-2, 5B-2, and 8B). Fortunately, this incompleteness of the reaction is advantageous for detecting the prospective single peak of C-terminal peptide amides not exhibiting the mass shift of 13 Da, which could otherwise be discerned as the appearance of a pair of peaks that signify the existence of a C-terminal-free carboxyl group. In this study, we slightly modified the original protocol [10, 11] to increase the yield of methylamidation by adding Pfp-OH

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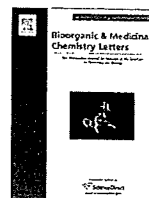

A method for terminus proteomics: Selective isolation and labeling of N-terminal peptide from protein through transamination reaction

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ABSTRACT

A novel method for selectively labeling and isolating N-terminal peptide from protein has been developed. An N^{α}-amino group of protein was converted to a carbonyl group through transamination reaction and the resulting carbonyl group was modified with *O*-(4-nitrobenzyl)hydroxylamine (NBHA). After proteolytic digestion using *Grifola frondosa* metalloendopeptidase (LysN), the modified N-terminal peptide remained unbound in the following treatment using amino-reactive *p*-phenylenediisothiocyanate (DITC) glass, whereas peptides other than the N-terminal peptide were effectively scavenged from the supernatant solution. The modified N-terminal peptide was thus successfully isolated and sequenced by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) analysis.

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Protein identification using mass spectrometry is a key technology in proteome research. In the conventional approach, such as peptide mass fingerprinting (PMF), target proteins are enzymatically or chemically digested into peptide fragments and measured mass values are compared with theoretical ones calculated from genome or protein databases.[1] Therefore, the exact state of mature proteins is not always determined; the mature protein sequence may often differ from that translated from its DNA sequence due to processes such as splicing and shuffling of mRNA, and/or the various post-translational modifications (PTMs). For conducting further study in depth on each protein identified by conventional proteome analysis, more detailed information about the exact amino acid sequence and PTMs of mature proteins from N-terminus to C-terminus has been highly desired.

Mass spectrometry has become a major tool in protein and peptide analysis and various methods have been developed and applied to actual samples.[2,3] However Edman sequencing is still the only conventional method for determining the N-terminal sequence. This method lacks generality, in that it cannot perform sequencing of N-terminally capped protein. Therefore, methodology using MS for both N- and C-terminal sequence analysis has been intensively studied for general and practical applications.[4–10]

We have been developing methods for isolating N- and C-terminal peptides from a peptide mixture of digested proteins and sequencing the isolated terminal peptides by MS.[8–10] In our review (in press), we proposed that this scientific field be termed 'terminus proteomics.'[11] In this study, we focused on the characteristic reactivity of metal-ion catalyzed transamination reaction,[12] which one of the authors (O.N.) had used for removing the N-terminal methionine residue from recombinant proteins produced from *Escherichia coli*.[13,14] This reaction selectively converts the N^{α}-amino group (N^{α}-NH_2) of peptides or proteins into a carbonyl group, whereas the coexisting N^{ε}-amino group (N^{ε}-NH_2) of lysine residue is kept intact. Reactions that selectively modify N^{α}-NH_2 or N^{ε}-NH_2 have often been utilized to isolate N- or C-terminal peptides from proteolytic digests; however, the selectivity is hardly obtained in general. The high selectivity of transamination reaction for N^{α}-NH_2 encouraged us to investigate its feasibility for isolating terminal peptides and we successfully developed a new method for C-terminal sequencing analysis using this transformation.[10] Here, we report an application of transamination reaction to N-terminal analysis, which was achieved by changing the enzyme used for digestion and by optimizing the procedure.

In the previous C-terminal analysis, proteins were first digested with lysylendopeptidase (LysC). LysC cleaves peptide bonds at the carboxyl side of lysine residues (-Lys-|-Xaa-) to yield mainly peptides having N^{α}-NH_2 and N^{ε}-NH_2 at both ends; however, C-terminal peptides have only N^{α}-NH_2. Subsequent transamination reaction converts the N^{α}-NH_2 to a carbonyl group; thus, incubation with amino-reactive *p*-phenylenediisothiocyanate (DITC) glass effectively scavenges N^{ε}-NH_2-containing peptides (peptides other than the C-terminal one). The recovered C-terminal peptide is open to modification with various nucleophilic reagents, such as hydrazine and hydroxylamine. We used 2,4-dinitrophenylhydrazine (DNPH), which demonstrated signal enhancement in MS analysis.

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In studying the C-terminal analysis employing a transamination reaction, we envisioned extending the method to N-terminal analysis by using *Grifola frondosa* metalloendopeptidase (LysN),[15–17] which cleaves proteins at the amino side of lysine residues, instead of LysC, as described in the previous report.[9] In addition, the protocol was changed so that a sample protein was first subjected to transamination and modification of the resulting carbonyl group, which facilitated the easy purification of the modified protein by simple gel filtration or ultrafiltration. Transamination reaction has been applied to proteins as well as peptides.[12,18–20] Thus, the procedure for N-terminal analysis was optimized (Scheme 1). The procedure consists of the following five steps: (1) transamination of proteins, (2) modification of the resulting carbonyl group through oxime formation, (3) LysN digestion, (4) treatment with DITC glass, and (5) MALDI -MS and MS/MS analysis. In this paper, sample proteins are transaminated and modified with *O*-(4-nitrobenzyl)hydroxylamine (NBHA) at their N-termini.[21] NBHA has a nitrobenzene moiety as well as DNPH, which would be expected to increase sensitivity in MALDI-MS analysis by combining a matrix system developed for sensitive detection of 2-nitrobenzenesulfenyl(NBS)-modified peptides.[22] LysN cleaves peptide bonds at the amino side of lysine residues (-Xaa-|-Lys-). After LysN digestion, the modified N-terminal peptide contains no amino group. Amino-reactive DITC glass scavenges the peptide fragments other than the N-terminal peptide, and the target peptide is left unreacted in the supernatant. Thus recovered N-terminal peptides are analyzed by MALDI-MS and MS/MS.

Two model proteins, bovine α-lactalbumin (α-la) and bovine β-lactoglobulin (β-lg), were chosen for testing the protocol. Proteins were transaminated in 10% pyridine containing 0.2 M glyoxylic acid, 6 mM $CuSO_4$, and 2 M urea. It was reported that the transamination reaction proceeded as well in the presence of urea.[12,14] Reaction time was set to 1 h, based on the results of our preliminary experiments using some peptides incorporating a different residue at their N-termini (data not shown). The reaction mixture was then subjected to a buffer exchange into 100 mM phosphate (pH 6.0) containing 2 M urea by ultrafiltration. Subsequent modification with NBHA was performed in 100 mM phosphate (pH 6.0) containing 50 mM NBHA-HCl and 2 M urea for 2 h at 37 °C. After a buffer exchange into 50 mM $NaHCO_3$ containing 2 M urea by ultrafiltration, disulfide bonds were reduced with tris(2-carboxyethyl)phosphine hydrochloride and the resulting free sulfhydryl groups were alkylated with iodoacetamide. LysN digestion was then performed at an enzyme-to-substrate ratio of 1:40 at room temperature for 15 h.[9] The upper panels in Figure 1 depict MALDI-MS spectra[23] after LysN digestion (Fig. 1a for α-la and Fig. 1c for β-lg), in which the modified N-terminal peptides (indicated by arrows) were detected among the peptide fragments as a sodium adduct ($[M+Na]^+$: 661.3 from α-la and $[M+Na]^+$: 976.5 from β-lg). When peptides containing a nitrobenzene moiety are analyzed by MALDI-MS, they are often accompanied by peaks with a 16 Da decrease probably due to splitting off of the oxygen atom from nitro group.[22] In both spectra these peaks were detected

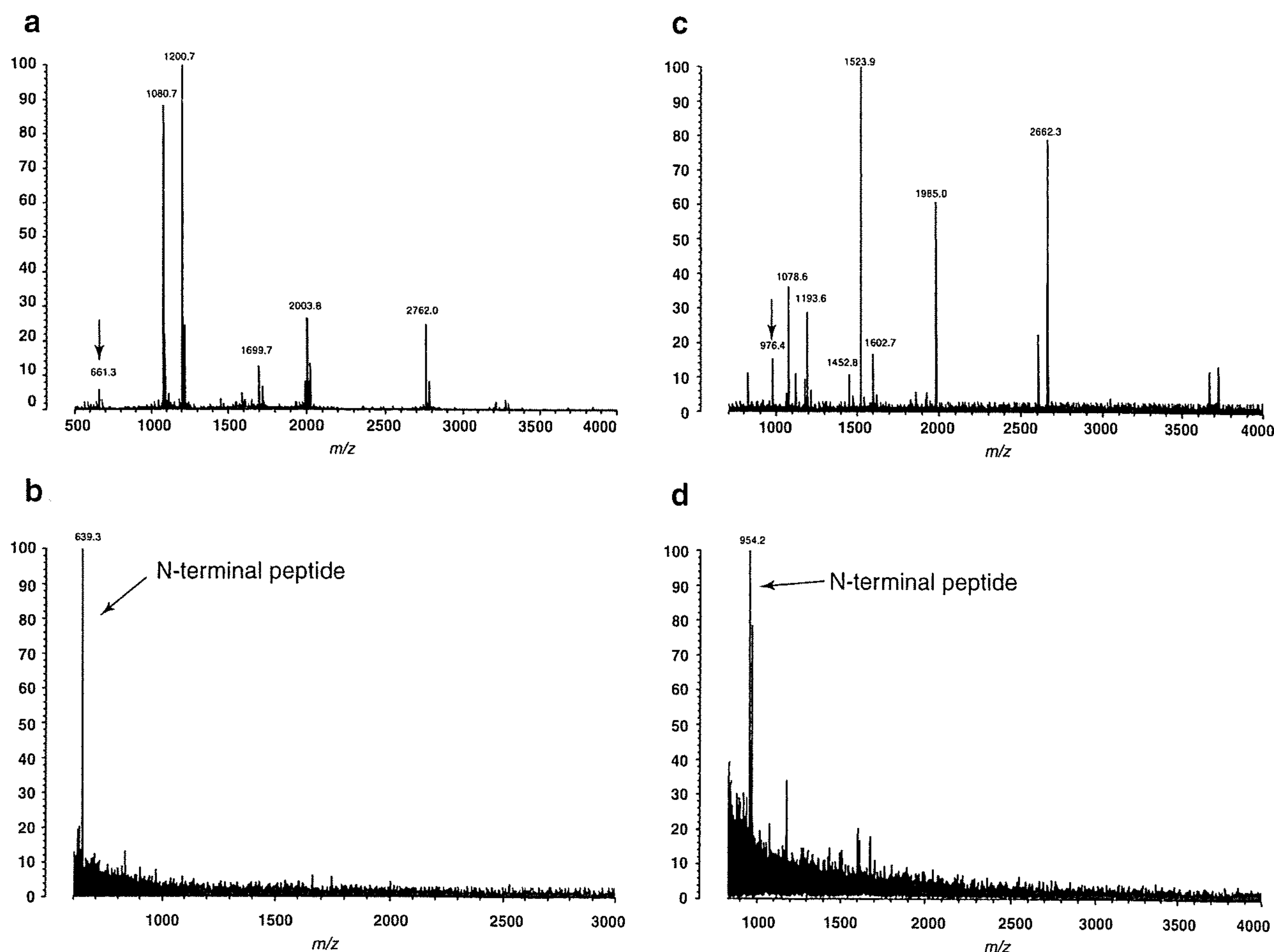


Figure 1. MALDI-MS spectra of peptide fragments of model proteins after LysN digestion ((a) α-lactalbumin, (c) β-lactoglobulin) and after treatment with DITC glass ((b) α-lactalbumin, (d) β-lactoglobulin.). Arrows indicate the modified N-terminal peptides of each protein. An aliquot (3 pmol of digest) was loaded onto the target plate.

and aided in discriminating the modified N-terminal peptides from the other peptide fragments even before the isolation step.

The LysN digest was then treated with DITC glass.[9,24] An aliquot of the digest (30 pmol) was added to pre-washed DITC glass. After 2 h incubation at 60 °C, the supernatant was directly analyzed by MALDI-MS. We employed a mixture of α-cyano-4-hydroxycinnamic acid (CHCA) and 3-hydroxy-4-nitrobenzoic acid (3H4NBA) as a binary matrix system, which was used for sensitive detection of NBS-modified peptides and was shown to be applicable to peptides containing a nitrobenzene moiety.[22,25] The lower panels in Figure 1 illustrate the MALDI-MS spectra after the DITC treatment (Fig. 1b for α-la and Fig. 1d for β-lg). Each N-terminal peptide was singly recovered ($[M+H]^+$: 639.3 from α-la and $[M+H]^+$: 954.5 from β-lg). In this experiment, these modified N-terminal peptides needed 3H4NBA as a matrix for the detection, the usefulness of which was also demonstrated in this case.

The isolated N-terminal peptides were subjected to MALDI-MS/MS analysis in the post-source decay (PSD) mode. The amino acid sequences of the peptides were analyzed using the observed fragment peaks (Fig. 2a for α-la and Fig. 2b for β-lg).

For N-terminally acetylated proteins, the method described here can be employed for N-terminal analysis because the N-terminal acetyl group is not affected in the transamination reaction (data not shown) and the acetylated N-terminal peptide after LysN digestion is free from the amino group. Studies in the case of N-blocked proteins were described in the previous reports.[5,6,9,26]

In the present study, we developed N-terminal analysis of proteins through transamination reaction. The method was applied to two model proteins, and successful isolation and sequencing of the N-terminal peptides were demonstrated. This method can not be used for some proteins (e.g., proteins containing N-terminal proline) because of limited reactivity for transamination.[10,27] However, an α-carbonyl of N-terminal ketoacyl group generated after transamination reaction can react with various types of nucleophilic reagents, and virtually any functionality can be introduced into the N-terminus of the peptide. This flexibility is advantageous for the MALDI-MS analysis of terminal peptides whose sensitivity largely depends on its amino acid composition, size, and modification states.

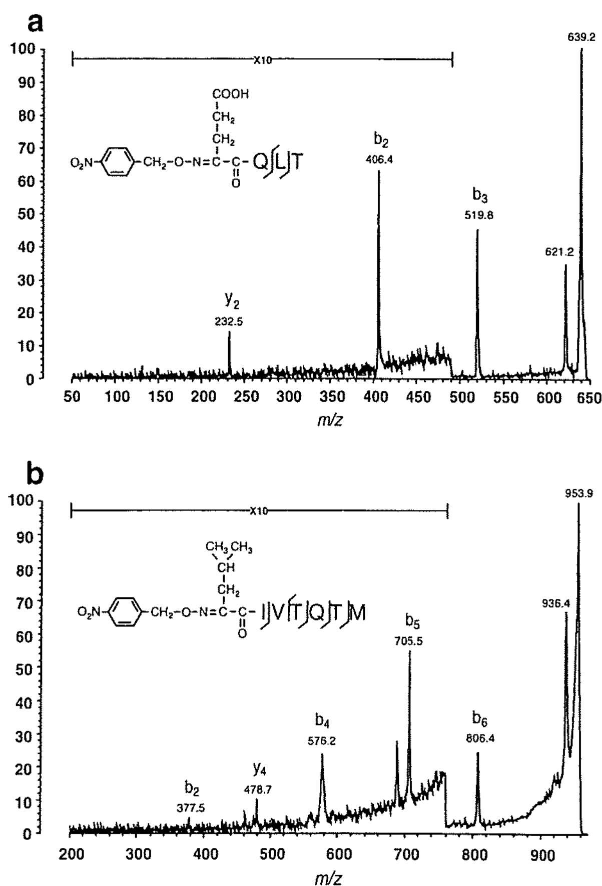
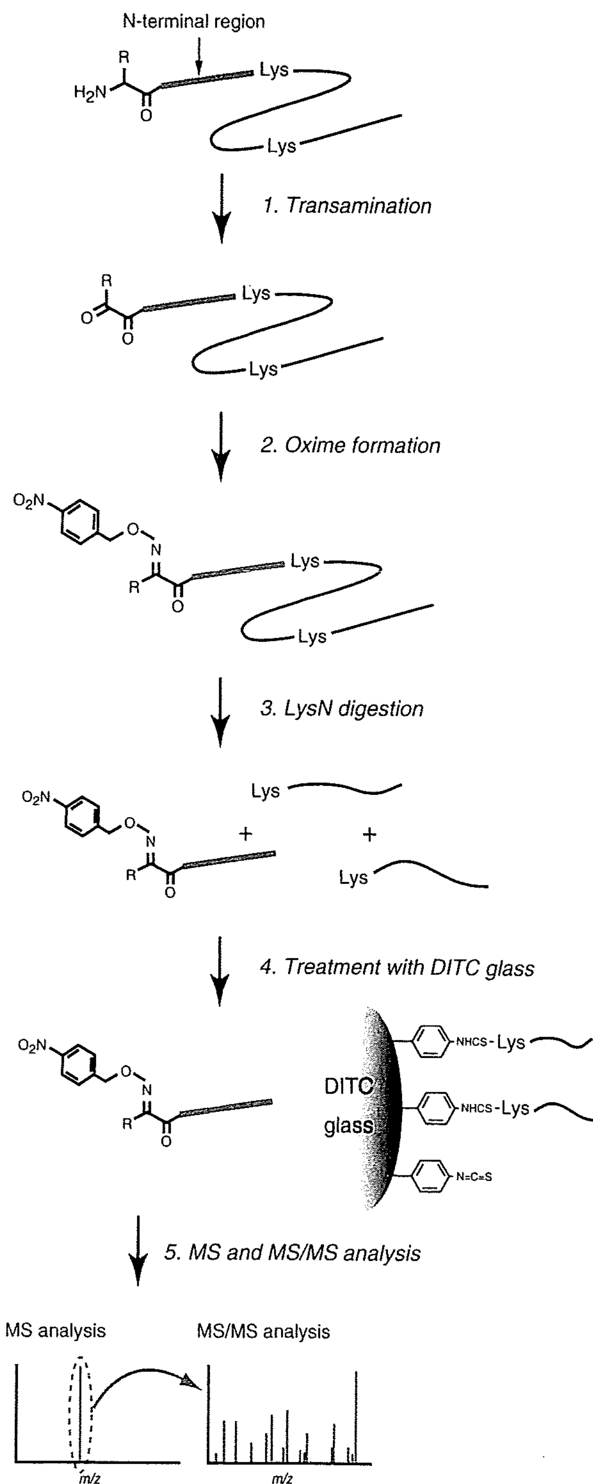


Figure 2. MALDI-MS/MS spectra (PSD mode) of the isolated N-terminal peptides of model proteins. (a) α-lactalbumin, (b) β-lactoglobulin.



Scheme 1. Protocol for selectively labeling and isolating N-terminal peptides.

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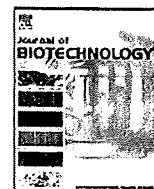

Expression of human Cu, Zn-superoxide dismutase in an insect cell-free system and its structural analysis by MALDI-TOF MS

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ABSTRACT

Human Cu, Zn-superoxide dismutase (hSOD1) is a homodimer that coordinates one copper and one zinc ion per monomer. These metal ions contribute to its enzymatic activity and structural stability. In addition, hSOD1 maintains an intra-subunit disulfide bond formed in the reducing environment of the cytosol and is active under a variety of stringent denaturing conditions. We report the expression of hSOD1 in a cell-free protein synthesis system constructed from *Spodoptera frugiperda* 21 (Sf21) insect cells, and its structural analysis including the status of the sole intra-subunit disulfide bond by mass spectrometry. By using this system hSOD1 was obtained in a soluble active form after addition of Cu^{2+} and Zn^{2+} and was purified with a yield of approximately 33 μg from 1 ml of reaction volume. Both enzymatic and structural analyses of the recombinant hSOD1 indicate that it was completely identical to the protein isolated from human erythrocytes.

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1. Introduction

A large number of proteins require metal ions for their enzymatic activity and structural stability. Typical metals and their order of abundance in living organisms are iron, zinc, and copper, etc., and zinc ions are especially important for various proteins involved in diverse cellular processes. A recent bioinformatics report states that 2800 proteins corresponding to 10% of the human proteome are potentially zinc-binding (Andreini et al., 2006).

Cell-free protein synthesis systems allow us to optimize the reaction condition for each targeted protein by the addition of proper reagents. Therefore it is suitable for the synthesis of a protein that requires a co-factor for its enzymatic activity and/or structural stabilization. There are several reports regarding the expression of metalloproteins such as a heme protein from *Phanerochaete* (Miyazaki-Imamura et al., 2003) and a zinc-binding protein from *Arabidopsis* (Matsuda et al., 2006) using an *Escherichia coli* cell-free system, and it was reported that the proper concentration of metal ions in the reaction increased the solubility and yield of targeted metalloproteins. Though the heme protein was expressed in an active form after addition of hemin and various kinds of molecular chaperones, its structural analysis was not reported. Whereas the expression and characterization of plant-specific zinc-binding transcriptional factors have been well investigated, only the expression of the DNA binding domains, but not of the entire mature proteins, has been described.

A cell-free protein synthesis system (Transdirect *insect cell*) derived from Sf21 insect cells (Ezure et al., 2006; Suzuki et al., 2006a) has been developed as a tool for post-genomic studies to improve the efficiency of producing targeted proteins, especially in cases where it is difficult to obtain sufficient amounts for analyses, including measurement of enzymatic activity, western blotting and investigation of post-translational modifications such as N-terminal protein modifications (Suzuki et al., 2006b), protein prenylation (Suzuki et al., 2007) and formation of disulfide bonds (Ezure et al., 2007) by MS. Other post-translational modifications such as formation of protein complexes (Masuda et al., 2007) and ubiquitination (to be published elsewhere) also occurred in the insect cell-free system. In addition, core glycosylation and cleavages of signal peptides were observed after the addition of microsomal membranes to the reaction mixture (unpublished data). However, acquisition of metal ions has not been successful in this system. Therefore this insect cell-free system was used to examine the conditions required for optimal translation of hSOD1 as a model metalloprotein. hSOD1 exists in various organism and scavenges superoxide radicals to protect cells against oxidative stress. hSOD1 is a homodimeric enzyme that coordinates one copper and one zinc ion per monomer (McCord and Fridovich, 1969;

Abbreviations: CAT, chloramphenicol acetyltransferase; FALS, familial amyotrophic lateral sclerosis; hSOD1, human Cu, Zn-superoxide dismutase; PDI, protein disulfide isomerase; PMF, peptide mass fingerprinting; Sf21, *Spodoptera frugiperda* 21.

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Forman and Fridovich, 1973; Briggs and Fee, 1978; Roe et al., 1988); the structural identity of recombinant hSOD1 was analyzed by MS and its structure and activity were compared with hSOD1 prepared from human erythrocytes. The present report demonstrates the expression of a metalloprotein in its mature form in a cell-free system and provides a detailed functional and structural analysis.

2. Materials and methods

2.1. Materials

Transdirect *insect cell* is a commercial product of Shimadzu (Kyoto, Japan). Restriction endonucleases and DNA modifying enzymes were purchased from TOYOBO (Osaka, Japan) and New England Biolabs, Inc. (Ipswich, MA). Desthiobiotin, TFA, CHCA and human SOD were from SIGMA (St. Louis, MO). *Strep*-Tactin superflow was from QIAGEN (Düsseldorf, Germany). Human cDNA clone hSOD1 (GenBank accession no. NM_000454) was obtained from TOYOBO. FluoroTect $Green_{Lys}$ tRNA was from Promega (Madison, WI).

2.2. Construction of plasmid

The expression plasmid for hSOD1 synthesis (pTD1-strep-hSOD1) was constructed as follows. The hSOD1 gene was amplified by PCR using the SOD-N primer (5′-ATGGCGACGAAGGCCG-3′) as the sense primer, the SOD-C primer (5′-GGGGTACCTTTTTGGGCGATCCCAATTACA-3′) as the antisense primer, and hSOD1 cDNA as the template. The amplified DNA fragment was then treated with T4 polynucleotide kinase. After digestion with *KpnI*, the amplified fragment was subcloned into the *Eco*RV-*KpnI* sites of a pTD1-strep vector (Ezure et al., 2007), and the resulting vector, pTD1-strep-hSOD1, containing hSOD1 having a *Strep*-tag at its C-terminus, was constructed.

For C6S/C111S double mutant synthesis, the expression plasmid (pTD1-strep-hSOD1-C6S/C111S) was constructed as follows. First, pTD1-strep-hSOD1-C6S was amplified by inverse PCR using the C6S-F primer (5′-GGCGACGGACCAGTGCAGG-3′) as the sense primer, the C6S-R primer (5′-CTTCAGCACGCTCACGGCCTT-3′) as the antisense primer, and pTD1-strep-hSOD1 as the template. After treatment with T4 polynucleotide kinase, the amplified fragment was allowed to self-ligate. Next, pTD1-strep-hSOD1-C6S/C111S was amplified by inverse PCR using the C111S-F primer (5′-TCAATCATTGGCCGCACACTGGT-3′) as the sense primer, the C111S-R primer (5′-ATGGTCTCCTGAGAGTGAGATCACA-3′) as the antisense primer, and pTD1-strep-hSOD1-C6S as the template. After treatment with T4 polynucleotide kinase, the amplified fragment was allowed to self-ligate.

pTD1-CAT (chloramphenicol acetyltransferase) was constructed using conventional cloning techniques. The DNA sequences of these recombinant constructs were confirmed by the dideoxynucleotide chain termination method.

2.3. Expression and fluorescent labeling of CAT

The mRNA was transcribed from pTD1-CAT and purified as described previously (Ezure et al., 2006). Cell-free protein synthesis was carried out using Transdirect *insect cell* in the presence over 0, 50, 100, 200, 400 and 800 μM $Cu(OAc)_2/Zn(OAc)_2$. Fluorescent labeling of *in vitro* translated proteins was carried out using FluoroTect $Green_{Lys}$ tRNA (Promega). 1 μl of FluoroTect was added to 50 μl of the reaction mixture. The reactions were carried out at 25 °C for 5 h. After the translation, an aliquot of the total fraction was centrifuged at 15,000 rpm for 15 min, and a supernatant fraction was obtained. 6 μl of these fractions were electrophoresed on 12.5% SDS-PAGE. The fluorescently labeled proteins were detected using a laser-based fluorescent scanner, Molecular Imager FX (Bio-Rad, Hercules, CA).

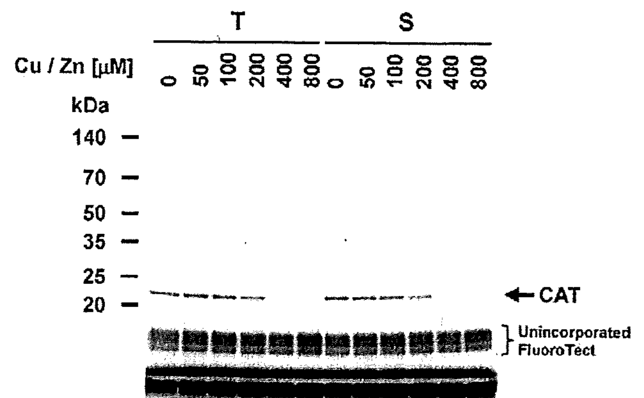

Fig. 1. SDS-PAGE analysis of CAT synthesized in the insect cell-free system. CAT was synthesized in the presence over 0, 50, 100, 200, 400 and 800 μM $Cu(OAc)_2/Zn(OAc)_2$. Fluorescent labeling of *in vitro* translated proteins was carried out as described in Section 2. 6 μl of the total (T) and supernatant (S) fractions were electrophoresed on 12.5% SDS-PAGE.

2.4. Expression and purification of hSOD1 and the hSOD1-C6S/C111S mutant

The mRNAs were synthesized and purified as described above. Cell-free protein synthesis was carried out at a 1 ml scale using Transdirect *insect cell* with or without the simultaneous addition of $Cu(OAc)_2$ and $Zn(OAc)_2$ to prepare equal final concentrations of Cu^{2+} and Zn^{2+} over the range of 1–400 μM. The reactions were carried out at 25 °C for 5 h, and the synthesized proteins were purified as described previously (Ezure et al., 2007). The proteins thus obtained were stored at −20 °C until use.

2.5. Measurement of enzymatic activity

SOD activity was determined using the SOD assay kit WST (Dojindo, Kumamoto, Japan) (Ukeda et al., 2002). One unit of SOD activity was defined as the amount of enzyme that inhibits the reaction of WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-

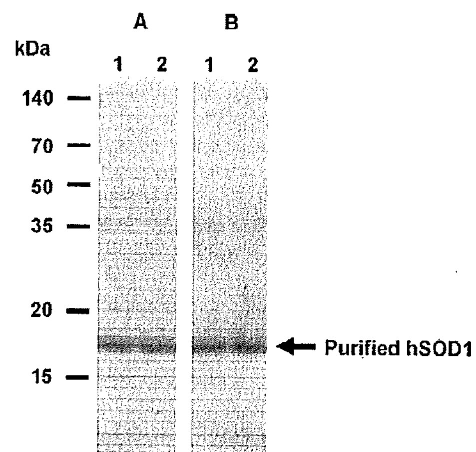

Fig. 2. SDS-PAGE analysis of purified wild-type and C6S/C111S mutant hSOD1s synthesized in the insect cell-free system under optimal conditions. The synthesized proteins were purified using a *Strep*-Tactin superflow column as described previously (Ezure et al., 2007). The purified proteins (1 μg) were electrophoresed on 15% SDS-PAGE under (A) reducing and (B) non-reducing conditions. Lanes 1 and 2: wild-type and C6S/C111S mutant hSOD1s, respectively.

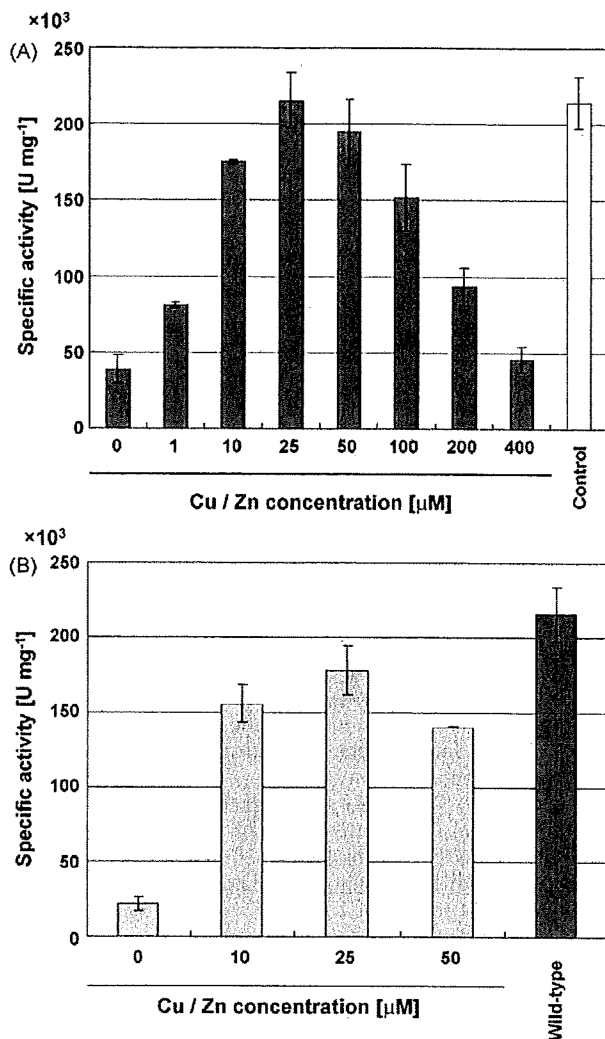

Fig. 3. Specific activities of wild-type (A) and C6S/C111S mutant (B) hSOD1s synthesized in the insect cell-free system after simultaneous addition of various concentrations of Cu/Zn ions. Control was the authentic hSOD1 purified from human erythrocytes, and wild-type indicates the hSOD1 synthesized in the cell-free system under optimal conditions. The means and standard deviations of three replicate experiments are indicated.

tetrazolio]-1,3-benzene disulfonate sodium salt) with superoxide anion by 50% at 37 °C for 20 min.

2.6. *Protein assay*

The protein concentration was quantified using the QuantiPro™ BCA Assay Kit (SIGMA). Bovine serum albumin was used as the standard protein.

2.7. *Confirmation of amino acid sequence and disulfide status of hSOD1 by MALDI-TOF MS*

The purified wild-type and C6S/C111S mutant hSOD1s (each 10 μg) were denatured in 10 μl of 8 M urea solution and then submitted to one of the three following conditions: (a) reduction with DTT followed by *S*-alkylation with iodoacetamide, (b) *S*-alkylation with iodoacetamide and (c) no treatment. Each reaction mixture was diluted by adding 90 μl of 50 mM ammonium bicarbonate and was then digested with trypsin (350 ng, Promega) overnight. The resulting tryptic digests were desalted and concentrated to approximately 8 μl by ZipTip μ-C18 (Millipore, Billerica, MA). Aliquots of samples (each 0.5 μl) were each mixed with 0.5 μl of CHCA solution (5 mg ml^{-1} in 50% (v/v) acetonitrile containing 0.1% (v/v) TFA) on the MALDI target plate and analyzed. The mass spectra of the tryptic digests were acquired in reflectron positive ion mode with an AXIMA-CFR™-plus MALDI-TOF MS instrument (Shimadzu/Kratos, Manchester, UK) according to a standard method (Yamaguchi et al., 2005).

3. Results and discussion

3.1. *Effect of metal ions on the insect cell-free protein synthesis*

Matsuda et al. (2006) synthesized CAT in the presence of zinc ion to confirm the effect of zinc ion on an *E. coli* cell-free system. For the same purpose, CAT was synthesized in the insect cell-free system by simultaneously adding equal amounts of $Cu(OAc)_2$ and $Zn(OAc)_2$ utilizing a fluorescence labeling method, and the yield and solubility of the resulting proteins were analyzed by SDS-PAGE. As the concentrations of both metal ions were increased, CAT synthesis was gradually inhibited and its yield decreased (Fig. 1). A similar phenomenon was observed using an *E. coli* cell-free system (Matsuda et al., 2006). Taking into consideration the fact that the concentrations of metal ions such as Cu^{2+} and Zn^{2+} have little effect in general on protein solubility, this suggests that these ions might have essentially inhibitory effects on the cell-free protein synthesis.

3.2. *Expression, purification and characterization of hSOD1*

Wild-type hSOD1 proteins synthesized at various concentrations of metal ions were purified by affinity column chromatography, and their purities were judged by SDS-PAGE with or without reducing reagent followed by staining with CBB (Fig. 2, lane 1). Each purified protein ran as nearly a single band having a molecular mass of about 18 kDa, regardless of the presence or absence of a reducing reagent. Each apparent molecular mass was compatible with the theoretical value calculated from the amino acid sequence of hSOD1. These results suggest that hSOD1 proteins expressed in this cell-free system do not form an inter-subunit disulfide bond as in the authentic hSOD1, despite the existence of two sulfhydryl groups per subunit.

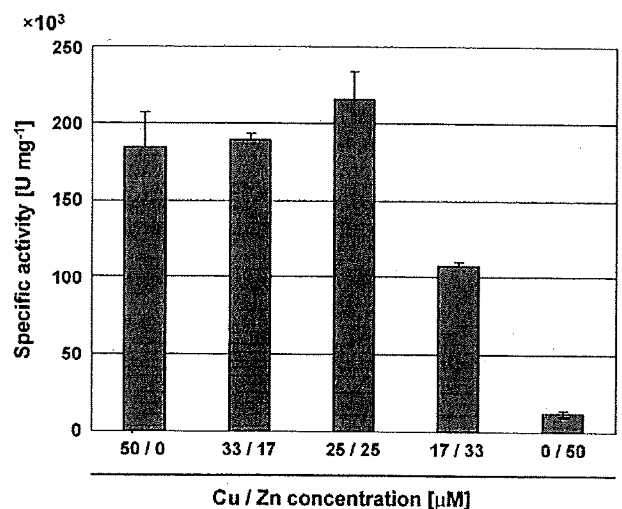

Fig. 4. Specific activities of wild-type hSOD1 synthesized in the insect cell-free system after simultaneous addition of various ratios of Cu/Zn ions. Total concentration of Cu^{2+} and Zn^{2+} was set at 50 μM. The means and standard deviations of three replicate experiments are indicated.

(M)1ATKAVCVLKG DGPVQGIINF EQKESNGPVK VWGSIKGLTE GLHGFHVHEF50
51GDNTAGCTSA GPHFNPLSRK HGGPKDEERH VGDLGNVTAD KDGVADVSIE100
101DSVISLSGDH CIIGRTLVVH EKADDLGKGG NEESTKTGNA GSRLACGVIG150
151IAQKGTGSGG GGGGGWSHP QFEK
Spacer sequence Strep-tag sequence

Fig. 5. Amino acid sequence of the authentic hSOD1 and the location of its disulfide bond. Disulfide linkage of hSOD1 is indicated by a line. Spacer and *Strep*-tag sequences are underlined. The regions which were not detected in the PMF analysis of wild-type hSOD1 are indicated by a dotted line.

Similar to the expression of CAT, the yields of hSOD1 proteins depended on the concentrations of metal ions. The simultaneous addition of $Cu(OAc)_2$ and $Zn(OAc)_2$ at final concentrations of 200–400 μM significantly decreased the yields of hSOD1s (data not shown), whereas by simultaneous addition of $Cu(OAc)_2$ and $Zn(OAc)_2$ at final concentrations of 25 μM, hSOD1 produced the highest specific activity (Fig. 3A). This value ($215{,}788.7 \pm 17{,}965.8$ units mg^{-1}) was almost equal to that of the authentic hSOD1 protein ($214{,}188.7 \pm 16{,}915.0$ units mg^{-1}), which was produced with a final yield of 33 μg from 1 ml of the reaction mixture. It has been reported that for hSOD1 synthesis using *in vivo* expression systems such as *E. coli* (Hartman et al., 1986), yeast (Hallewell et al., 1987) and baculovirus (Fujii et al., 1995), the concentration of Cu^{2+} affects only the yield of the active form of the protein, because the medium contains sufficient Zn^{2+}. To confirm the optimum ratio of added Cu^{2+} and Zn^{2+} in the insect cell-free system, hSOD1 proteins were synthesized at various ratios of metal ions. Total concentration of Cu^{2+} and Zn^{2+} was set at 50 μM. The optimal ratio of added Cu^{2+} and Zn^{2+} was 1:1 (Fig. 4), suggesting that the optimal ratio of Cu^{2+} and Zn^{2+} corresponds to that incorporated into the targeted protein, because metal ion concentration were controlled in the cell-free system. Whereas 85.5% of the specific activity at the optimum ratio was observed by only addition of $Cu(OAc)_2$ at final concentration of 50 μM, hSOD1 produced low activity by only addition of $Zn(OAc)_2$. These results indicated that cupper plays the main role of hSOD1 activity as reported (Rae et al., 2001). Furthermore, there are several reports regarding the reconstitution of hSOD1 in its apo-form (Jewett et al., 1982; Yamazaki and Takao, 2008). Though this method might be suitable for stable proteins such as hSOD1, it should be emphasized that the co-translational method described here is very useful for synthesis of various eukaryotic metalloproteins in the proteomic era.

3.3. Confirmation of the amino acid sequence of hSOD1 synthesized in the insect cell-free system and location(s) of disulfide bond(s)

To confirm the amino acid sequence and location(s) of the disulfide bond(s) of wild-type hSOD1 synthesized in the insect cell-free system, the protein was analyzed by MALDI-TOF MS. Fig. 5 shows the location of a disulfide bond for the authentic protein along with its complete amino acid sequence. The purified recombinant protein was treated under one of the three following conditions: (a) reduction followed by *S*-alkylation with iodoacetamide, (b) *S*-alkylation with iodoacetamide, and (c) no treatment, followed by tryptic digestion. Resulting tryptic peptides from each treatment were analyzed by MALDI-TOF MS. The peptide mass fingerprinting (PMF) for each protein sample was identical, with the exception of regions corresponding to cysteine-containing peptides, regardless of sample treatment conditions, and the values obtained were in good agreement with the theoretical values calculated from the authentic protein assuming the presence of a disulfide bond between residues 57 and 146, plus two carbamidomethyl-cysteines at positions 6 and 111 resulting from *S*-alkylation treatment using iodoacetamide (Table 1). Thus, 75.9% of the sequence coverage on PMF was observed for each purified protein sample, i.e. the amino acid sequence of 132 of the total 174 residues (Fig. 5). This result strongly indicates that the protein is identical to the authentic hSOD1.

For protein samples treated with *S*-alkylation, regardless of reduction, three peaks that apparently corresponded to tryptic fragments containing carbamidomethyl-cysteine were observed at *m/z* values 2171.87, 2514.33 and 3720.94. These values were in good agreement with the theoretical values (*m/z* 2172.15, 2514.21, 3720.81) calculated from the amino acid sequence of the authentic protein. Furthermore, for the protein that was not treated

Table 1
Theoretical and observed monoisotopic mass values for tryptic digests of hSOD1.

<table>
<tr><th rowspan="2">Mass value</th><th rowspan="2">Position</th><th rowspan="2">Modification(s)</th><th rowspan="2">Modified mass value</th><th colspan="3">Observed mass value[a]</th><th rowspan="2">Peptide sequence</th></tr>
<tr><th>(a)</th><th>(b)</th><th>(c)</th></tr>
<tr><td>632.38</td><td>4–9</td><td>Cys_CAM[c]: 6</td><td>689.40</td><td colspan="2">689.43</td><td>632.38</td><td>AVCVLK</td></tr>
<tr><td>1501.76</td><td>10–23</td><td></td><td></td><td colspan="3">1501.89</td><td>GDGPVQGIINFEQK</td></tr>
<tr><td>2115.13</td><td>4–23 (MC[b]:1)</td><td>Cys_CAM: 6</td><td>2172.15</td><td colspan="2">2171.87</td><td>2115.44</td><td>AVCVLKGDGPVQGIINFEQK</td></tr>
<tr><td>730.37</td><td>24–30</td><td></td><td></td><td colspan="3">N.D.[d]</td><td>ESNGPVK</td></tr>
<tr><td>689.40</td><td>31–36</td><td></td><td></td><td colspan="3">689.43</td><td>VWGSIK</td></tr>
<tr><td>3462.60</td><td>37–69</td><td>Cys_CAM: 57</td><td>3519.62</td><td>3519.74</td><td colspan="2">N.D.</td><td>GLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSR</td></tr>
<tr><td>1225.62</td><td>80–91</td><td></td><td></td><td colspan="3">1225.71</td><td>HVGDLGNVTADK</td></tr>
<tr><td>2457.19</td><td>92–115</td><td>Cys_CAM: 111</td><td>2514.21</td><td colspan="2">2514.33</td><td>2457.49</td><td>DGVADVSIEDSVISLSGDHCIIGR</td></tr>
<tr><td>3663.79</td><td>80–115 (MC:1)</td><td>Cys_CAM: 111</td><td>3720.81</td><td colspan="2">3720.94</td><td>3663.93</td><td>HVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGR</td></tr>
<tr><td>825.48</td><td>116–122</td><td></td><td></td><td colspan="3">825.53</td><td>TLVVHEK</td></tr>
<tr><td>618.31</td><td>123–128</td><td></td><td></td><td colspan="3">N.D.</td><td>ADDLGK</td></tr>
<tr><td>821.36</td><td>129–136</td><td></td><td></td><td colspan="3">N.D.</td><td>GGNEESTK</td></tr>
<tr><td>662.32</td><td>137–143</td><td></td><td></td><td colspan="3">N.D.</td><td>TGNAGSR</td></tr>
<tr><td>1072.62</td><td>144–154</td><td>Cys_CAM: 146</td><td>1129.64</td><td>1129.73</td><td colspan="2">N.D.</td><td>LACGVIGIAQK</td></tr>
<tr><td>1816.80</td><td>155–174</td><td></td><td></td><td colspan="3">1816.97</td><td>GTGSGGGGGGGGWSHPQFEK</td></tr>
</table>

Mass values over 600 are shown.
[a] (a)–(c) stand for the sample conditions described in Section 2.
[b] MC stands for the number of missed cleavages.
[c] Cys_CAM stands for carbamidomethyl-cysteine.
[d] Not detected.

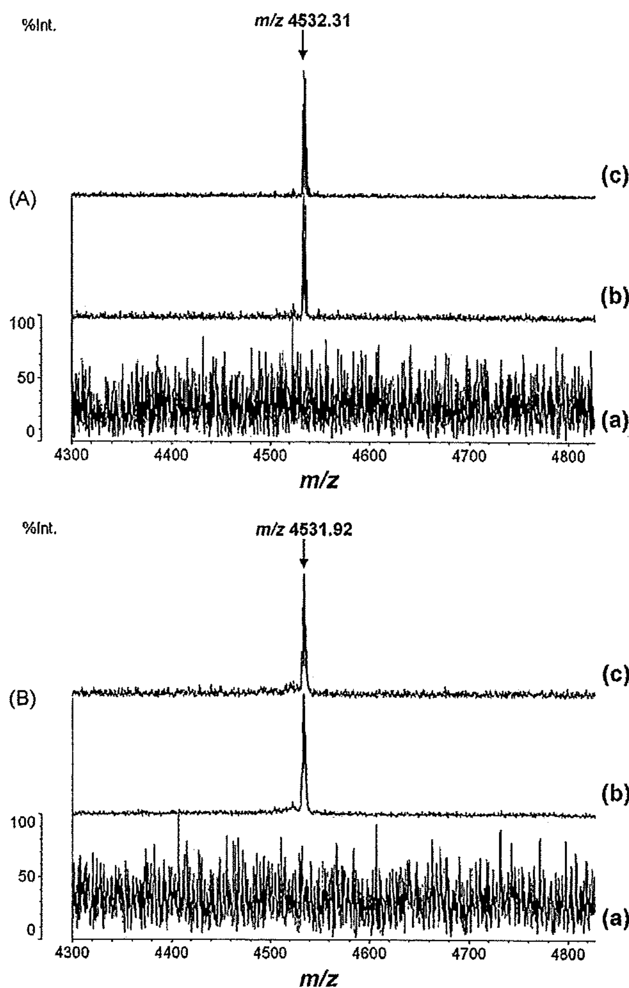

Fig. 6. MALDI-mass spectra of tryptic digests of wild-type (A) and C6S/C111S mutant (B) hSOD1s. The acquired profiles were focused on mass ranges from 4300 to 4800. The purified hSOD1s were treated under the following three conditions: (a) reduction and S-alkylation, (b) S-alkylation, and (c) no treatment. Arrow and numerical value indicate specific peptide peak and observed mass, respectively.

with S-alkylation, peaks having m/z values of 2115.44, 2457.49 and 3663.93, instead of the three peaks described above, were observed. Each value was in good agreement with the theoretical value (m/z 2115.13, 2457.19, 3663.79) calculated from the tryptic peptide fragments containing un-modified cysteines (corresponding to residue No.6 and No.111). The results clearly showed that Cys^{6} and Cys^{111} in the protein synthesized in this system did not form a disulfide bond.

On the other hand, in the case of the reduced and S-alkylated protein, two peaks were clearly observed at m/z values of 3519.74 and 1129.73, which were in good agreement with the theoretical values (3519.62 and 1129.64) assuming that each peptide contained a carbamidomethyl-cysteine at residue No. 57 and No. 146, respectively. However, these peaks were not detected for the non-reduced S-alkylated hSOD1 or for the untreated protein, and a peak at m/z value of 4532.31 was observed (Fig. 6A), which was in good agreement with the theoretical value (4532.22) calculated from a tryptic peptide predicted for a protein with a disulfide linkage between Cys^{57} and Cys^{146} (Table 2).

No peaks corresponding to predicted values for any other disulfide linkages were detected, indicating that the wild-type hSOD1 synthesized in the insect cell-free system has a highly conserved pair of cysteines (Cys^{57} and Cys^{146}) that form an intra-subunit disulfide bond as well as two free cysteines (Cys^{6} and Cys^{111}) similar to the hSOD1 from human erythrocytes (Lepock et al., 1990; Parge et al., 1992; Arnesano et al., 2004; Lindberg et al., 2004).

3.4. Enzymatic and structural analyses of the hSOD1 C6S/C111S mutant synthesized using the insect cell-free system

To confirm the contribution of two free cysteines (Cys^{6} and Cys^{111}) to the enzymatic activity of hSOD1 and the disulfide bond formation, a C6S/C111S double mutant, in which two free cysteines were replaced by serine, was constructed. The C6S/C111S mutant was synthesized and purified (Fig. 2, lane 2). As in the synthesis of wild-type hSOD1, the simultaneous addition of $Cu(OAc)_2$ and $Zn(OAc)_2$ at final concentrations of 25 μM Cu^{2+} and Zn^{2+} was optimal for the enzymatic activity of the C6S/C111S mutant (Fig. 3B), although its specific activity (178,335.3 ± 16,087.8 units mg^{-1}) was slightly less than that of the wild-type protein (215,788.7 ± 17,965.8 units mg^{-1}). However, MALDI-TOF MS analysis demonstrated that this mutant, when synthesized under the optimized conditions, formed a disulfide linkage between Cys^{57} and Cys^{146} similar to the wild-type hSOD1 (Fig. 6B). The results indicated that two free cysteine residues, Cys^{6} and Cys^{111}, had little effect on the SOD activity as reported (Parge et al., 1992; Arnesano et al., 2004), and that they were not involved in disulfide formation.

Recently, hSOD1 have attracted attention because familial amyotrophic lateral sclerosis (FALS) is possibly caused by the aggregation of hSOD1. It is suggested that some mutations in region of interface cause dissociation of a dimer to a monomer (Deng et al., 1993). A lot of mutations have been identified in patients with FALS. Therefore, the method of mutagenesis analysis described above could be useful tool for the preparation of hSOD1 mutants.

In order to become enzymatically active by forming the correctly folded quaternary structure, several post-translational modifications of hSOD1 are required, including acquisition of a 1:1 molar ratio of copper and zinc ions, respectively, formation of an intra-subunit disulfide bond, and dimerization. The insertion of copper by a copper chaperone for SOD1 is well established (Rae et al., 2001; Torres et al., 2001; Bartnikas and Gitlin, 2003; Furukawa et al., 2004), but the mechanism by which SOD1 acquires a zinc ion is not fully understood. The formation of a disulfide bond is a peculiar event for a cytosolic protein, because the cytosol is a strongly reducing environment. However, proteins containing disulfide bonds were expressed successfully using the insect cell-free system under

Table 2
Theoretical and observed monoisotopic mass values for disulfide-linked peptides.

Mass value	Position	MC[a]	Modification(s)	Modified mass value	Observed mass value	Peptide sequence[b]
3462.60	37–69	0	Disulfide bond: 57–146	4532.22	4532.31	GLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSR
1072.62	144–154	0				LACGVIGIAQK

[a] MC stands for the number of missed cleavages.
[b] Disulfide linkage is indicated by a line.

non-reducing conditions after addition of reduced glutathione, oxidized glutathione, and protein disulfide isomerase (PDI) in a previous study (Ezure et al., 2007). In the cell-free system using non-reducing conditions as described above, hSOD1 was synthesized but was insoluble, and its specific activity remained less than 15% that of the authentic hSOD1, even after addition of metal ions, and only a band corresponding to the monomer form was observed on SDS-PAGE analysis without a reducing reagent (data not shown). From these data, we concluded that a random inter-subunit disulfide bond could not be formed under these conditions. Normally, the formation and isomerization of disulfide bonds is catalyzed by PDI in the lumen of the endoplasmic reticula. In a previous study, this situation was reconstituted in the insect cell-free system. Because the secreted protein human lysozyme was used as a model protein in a previous study, it is supposed that this could be a suitable expression system. However, optimal expression conditions may differ according to the localization and disulfide status of targeted proteins.

In conclusion, the present study, by demonstrating that an active metalloprotein could be synthesized under optimized conditions using an insect cell-free protein synthesis system, shows that this system can effectively synthesize proteins that require metal ions for their correct quaternary structures and for their enzymatic activities.

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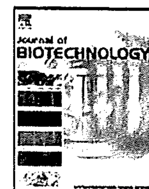

Preparation of ubiquitin-conjugated proteins using an insect cell-free protein synthesis system

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ABSTRACT

Ubiquitination is one of the most significant posttranslational modifications (PTMs). To evaluate the ability of an insect cell-free protein synthesis system to carry out ubiquitin (Ub) conjugation to *in vitro* translated proteins, poly-Ub chain formation was studied in an insect cell-free protein synthesis system. Poly-Ub was generated in the presence of Ub aldehyde (UA), a de-ubiquitinating enzyme inhibitor. *In vitro* ubiquitination of the p53 tumor suppressor protein was also analyzed, and p53 was poly-ubiquitinated when Ub, UA, and Mdm2, an E3 Ub ligase (E3) for p53, were added to the *in vitro* reaction mixture. These results suggest that the insect cell-free protein synthesis system contains enzymatic activities capable of carrying out ubiquitination. CBB-detectable ubiquitinated p53 was easily purified from the insect cell-free protein synthesis system, allowing analysis of the Ub-conjugated proteins by mass spectrometry (MS). Lys 305 of p53 was identified as one of the Ub acceptor sites using this strategy. Thus, we conclude that the insect cell-free protein synthesis system is a powerful tool for studying various PTMs of eukaryotic proteins including ubiqutination presented here.

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1. Introduction

There is increasing interest in analyzing PTMs of proteins. Cell-free protein synthesis systems are potentially powerful tools for post-genomic studies including analyses of PTMs, because they can not only be used to synthesize desired proteins, including those toxic to cells (Sakurai et al., 2007), but they can also carry out various PTMs on these proteins. A cell-free protein synthesis system from *Spodoptera frugiperda* 21 (Sf21) insect cells (Ezure et al., 2006), which are widely used as the host for baculovirus expression systems, was developed previously, and it was demonstrated that this system could generate various eukaryotic-specific protein modifications, such as *N*-myristoylation (Suzuki et al., 2006b) and prenylation (Suzuki et al., 2007).

Ubiquitination is one of the most significant PTMs because it plays central roles in the regulation of many cellular processes, such as targeting for proteasome degradation, cell cycle progression, signal transduction, DNA repair, and so on (Ciechanover, 1998; Hershko and Ciechanover, 1998). Therefore, techniques to prepare Ub-conjugated proteins are extremely important to understand these processes in detail. Some methodologies for the purification of ubiquitinated proteins have been developed (Tomlinson et al., 2007), and large-scale MS analyses have been performed (Peng et al., 2003; Gururaja et al., 2003). However, it is still challenging to identify ubiquitinated proteins and Ub-conjugation sites.

A rabbit reticulocyte lysate system has often been utilized for *in vitro* ubiquitination assays of target proteins, because it possesses enzymatic activities involved in the ubiquitination reaction (Ciechanover et al., 1991; Etlinger and Goldberg, 1977). However, a serious drawback of this system is that only radio-isotope labeling or an immunoblotting strategy may be used to detect ubiquitinated proteins, because of the low expression levels.

In order to evaluate whether the insect cell-free protein synthesis system contains enzymes capable of carrying out ubiquitination reactions, poly-Ub chain formation was investigated using the insect cell-free protein synthesis system and FLAG-tagged Ub. The p53 tumor suppresor protein was chosen as a model protein because it is highly regulated by the ubiquitin–proteasome pathway (Haupt et al., 1997), and *in vitro* ubiquitination of p53 occurred.

Abbreviations: PTMs, posttranslational modifications; Ub, ubiquitin; UA, ubiquitin aldehyde; E3, E3 ubiquitin ligase; MS, mass spectrometry; Sf21, *Spodoptera frugiperda* 21; Me-Ub, methylated ubiquitin; MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; QIT, quadrupole ion trap; MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio.

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The present study describes a simple and robust strategy to prepare ubiquitin-conjugated proteins using this cell-free protein synthesis system and the identification of exact location of Ub-conjugation sites by mass spectrometry.

2. Materials and methods

2.1. Materials

Transdirect *insect cell*, which is based on the Sf21 extract, is a commercial product of Shimadzu (Kyoto, Japan). Restriction endonucleases and DNA modifying enzymes were purchased from Toyobo (Osaka, Japan) and New England Biolabs (Ipswich, MA, USA). Human cDNA clone p53 (GenBank accession no. **NM_000546**) and Mdm2 (GenBank accession no. **BT007258**) were obtained from Toyobo and Open Biosystems (Huntsville, AL, USA), respectively. Caspase-3 Inhibitor I (Ac-DEVD-CHO) and MG-132 (Z-LLL-CHO) were obtained from Calbiochem (Darmstadt, Germany). Ubiquitin and FLAG-tagged ubiquitin were purchased from Sigma (St. Louis, MO, USA). Ubiquitin aldehyde and methylated ubiquitin were obtained from Peptide Institute (Osaka, Japan) and BostonBiochem (Cambridge, MA, USA), respectively.

2.2. Construction of expression clones for in vitro translation

The protein coding regions of human p53 were amplified by PCR and inserted into the multiple cloning site of the pTD1-strep vector, which is an expression vector for synthesizing C-terminal *Strep*-tagged target proteins using the insect cell-free protein synthesis system (Ezure et al., 2007). The resultant plasmid was named pTD1-strep-p53. The ORF of the human Mdm2 gene was amplified by PCR. The amplified DNA fragment was ligated into the pTD1-vector (Suzuki et al., 2006a), and the resulting plasmid was designated pTD1-Mdm2. N-terminal or C-terminal GST-tagged Mdm2 constructs were also constructed using conventional cloning techniques. The resultant plasmids were named pTD1-NGST-Mdm2 and pTD1-CGST-Mdm2, respectively. The DNA sequences of these recombinant constructs were confirmed by the dideoxynucleotide chain termination method.

2.3. In vitro transcription and translation

mRNAs were synthesized with the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA) using linearized expression clones as the template. Purification of *in vitro* transcribed mRNAs was performed as described previously (Suzuki et al., 2006b). *In vitro* translation was carried out using an insect cell-free protein synthesis system according to the instruction manual. In the case of Mdm2, translation was performed with or without the addition of a caspase-3 inhibitor I at a final concentration of 1.0 μM.

2.4. Detection of synthesized proteins by fluorescent labeling

For the synthesis of fluorescently labeled proteins, 1 μL of FluoroTect $Green_{Lys}$ tRNA (Promega) was added to 50 μL of the *in vitro* translation reaction mixture. The sample was resolved by SDS-PAGE. The fluorescently labeled proteins were detected using a laser-based fluorescent scanner, Molecular Imager FX (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Poly-Ub chain formation

The Sf21 cell-free extract for the insect cell-free protein synthesis system (75 μL) and the N-terminal FLAG-tagged Ub (50 μg) were mixed and incubated at 25 °C for 2 h in a 250 μL reaction mixture that included ubiquitination buffer (50 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate, 4 U mL^{-1} creatine kinase and 2.5 mM dithiothreitol) and 5 μg of ubiquitin aldehyde (UA).

2.6. In vitro ubiquitination assay

In vitro translated p53 (6 μL) and Mdm2 (4 μL) were mixed and incubated at 30 °C for 2 h in a 25 μL reaction mixture that included the ubiquitination buffer, 0.63 μg of UA, and 6.3 μg of Ub. Methylated Ub (Me-Ub) was added instead of Ub to the *in vitro* ubiquitination reaction mixture to suppress poly-Ub chain formation.

2.7. Affinity purification

Affinity purification of FLAG-tagged or strep-tagged proteins was performed as described previously (Suzuki et al., 2006b, 2007). In the case of GST-tagged proteins, a GST purification module was used (GE Healthcare, Piscataway, USA).

2.8. Analysis of N-terminal amino acid sequence

The affinity-purified protein was separated by SDS-PAGE and then transferred to a PVDF membrane, then stained with CBB R-250. The protein band was sequenced with a PPSQ-33A protein sequencer (Shimadzu).

2.9. Mass spectrometry

The tryptic digests from affinity-purified proteins were analyzed with an AXIMA-CFR-plus MALDI-TOF MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) instrument and an AXIMA-QIT MALDI-QIT (quadrupole IT)-TOF hybrid mass spectrometer (Shimadzu/Kratos, Manchester, UK) as described previously (Suzuki et al., 2006b).

3. Results

3.1. Poly-Ub chain formation using the insect cell-free extract

To evaluate the ability of the insect cell-free protein synthesis system to conjugate Ub to target proteins synthesized *in vitro*, generation of poly-Ub chains was analyzed after adding Ub to the cell-free extract of the insect cell-free protein synthesis system. FLAG-tagged Ub and the extract were incubated in the ubiquitination buffer, and then FLAG-tagged Ub was collected by affinity purification. The reaction was performed in the presence or absence of UA, a de-ubiquitinating enzyme inhibitor. Ladder bands at around 15–27 kDa, which probably corresponded to poly-Ubs, were observed upon SDS-PAGE of the affinity-purified sample after UA was added to the reaction mixture (Fig. 1). A protein band detected around 50 kDa was identified as β-tubulin by peptide mass fingerprinting (data not shown). This is probably a non-specific protein band because β-tubulin has been sometimes coeluted in the affinity purification step (Suzuki et al., 2007). On the other hand, when the reaction was carried out without adding UA, only a predominant 10 kDa band was detected (Fig. 1). The 10 kDa band and slowly migrating bands were excised individually and digested with trypsin, and the digests were analyzed by MALDI-TOF MS. The spectra produced from these samples were almost identical, and these MS spectra corresponded to tryptic digests of Ub (Fig. 2). Trypsin digestion of ubiquitinated proteins produces peptides with internal lysine residues harboring a di-glycine remnant (GG-tag)

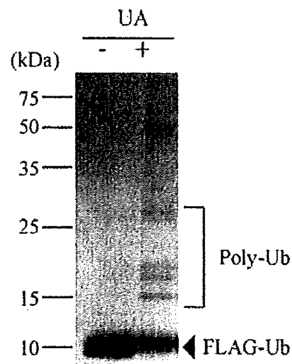

Fig. 1. Poly-Ub chain formation in the insect cell-free protein synthesis system. N-terminal FLAG-tagged Ub (50 μg) was added to the insect cell-free extract and incubated in the presence or absence of UA. After the incubation, FLAG-tagged Ub was purified as reported previously (Suzuki et al., 2007). The affinity eluate was concentrated to about 25 μL by ultrafiltration (molecular cutoff = 3 kDa). Five microliters of the concentrate was electrophoresed on a 15% SDS-PAGE gel and visualized by CBB staining.

derived from the C-terminus of Ub (Peng et al., 2003). Therefore, a search for ions corresponding to the tryptic peptide having a GG-tag was performed. Specific peaks that probably included a GG-tag were observed at *m/z* (mass-to-charge ratio) 1460.72, 1836.93, and 2244.09 in the MS spectra of tryptic digests of ladder bands (Fig. 2a). These ions were further subjected to tandem MS (MS/MS) analysis and identified as tryptic fragments of Ub containing a GG-tag on Lys 48, Lys 29, and Lys 63, respectively (Fig. 3), suggesting that the insect cell-free protein synthesis system contains enzymatic activities capable of carrying out ubiquitination and de-ubiquitination.

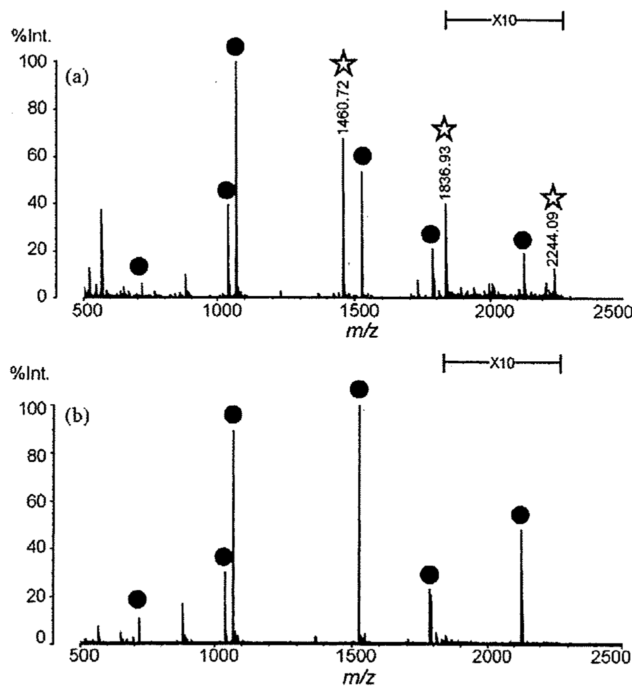

Fig. 2. MALDI-mass spectra of tryptic digests of the affinity-purified Ubs. The protein bands corresponding to (a) slowly migrating bands (around 15–27 kDa) and (b) 10 kDa were excised individually and digested with trypsin, and each digest was analyzed by MALDI-TOF MS. Filled circles indicate ions with the theoretical *m/z* values of tryptic digests of Ub. Stars indicate probable tryptic peptides containing a GG-tag.

3.2. In vitro ubiquitination of the p53 tumor suppressor using the insect cell-free protein synthesis system

To investigate whether the insect cell-free protein synthesis system has the ability to conjugate Ub to target proteins, the p53 tumor suppressor was chosen as a model protein. *In vitro* translation of mRNA encoding this protein was performed, and the *in vitro* ubiquitination assay was carried out as described in Section 2. A slight protein band probably corresponding to mono-ubiquitinated p53 was generated upon addition of both Ub and UA (Fig. 4: lane 4). To increase the efficiency of the p53 Ub-conjugation reaction, cell-free synthesized Mdm2, an E3 Ub ligase (E3) for p53 (Fang et al., 2000), was added to the *in vitro* ubiquitination reaction mixture. However, this produced no measurable effect (Fig. 4: lane 5).

To solve this problem, the *in vitro* synthesized Mdm2 was analyzed more closely, and it was noted that translation of the Mdm2 gene unexpectedly generated a 60 kDa protein band (Fig. 5), although the full-length predicted gene product of Mdm2 was

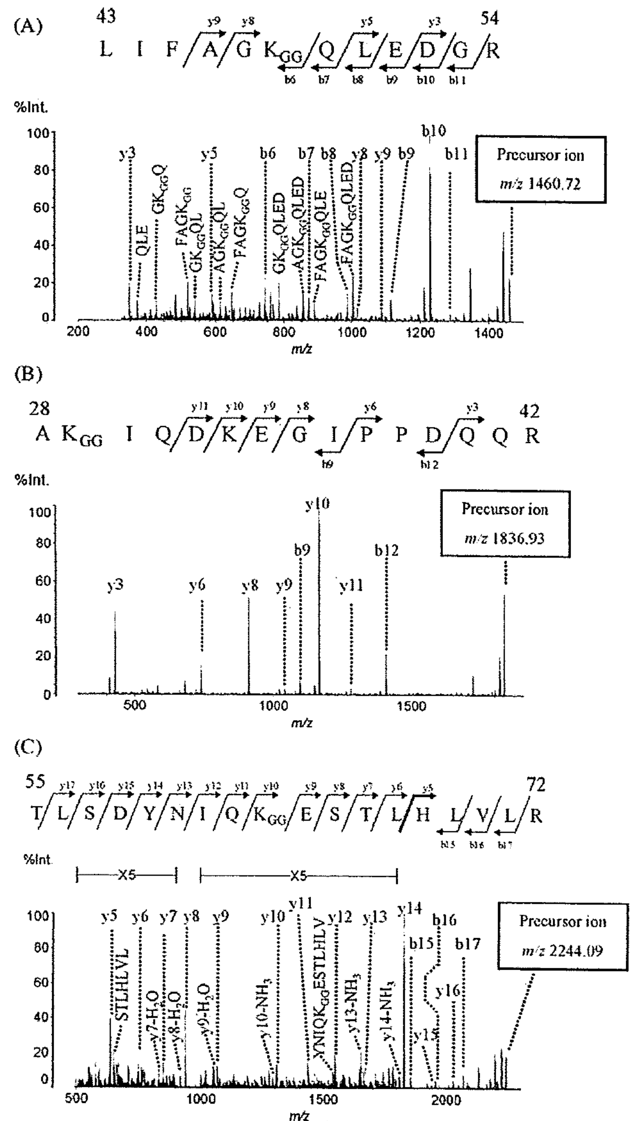

Fig. 3. MALDI-MS/MS spectra of the tryptic peptides containing a GG-tag derived from poly-Ub. MS/MS analyses were performed for the ions detected at *m/z* 1460.72 (A), *m/z* 1836.93 (B), and *m/z* 2244.09 (C) in the mass spectra of the tryptic digests from poly-Ub. The observed fragment ions are indicated by the sequences shown. The subscript "GG" represents di-glycine residues from the C-terminal region of Ub.