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A Method for the Detection of Asparagine Deamidation and Aspartate Isomerization of Proteins by MALDI/TOF–Mass Spectrometry Using Endoproteinase Asp-N

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A method was established for evaluating Asn deamidation and Asp isomerization/racemization. To detect the subtle changes in mass that accompany these chemical modifications, we used a combination of enzyme digestion by endoproteinase Asp-N, which selectively cleaves the N-terminus of L-α-Asp, and MALDI/TOF-mass spectrometry. To achieve better resolution, we employed digests of ^{15}N-labeled protein as an internal standard. To demonstrate the advantages of this method, we applied it to identify deamidated sites in mutant lysozymes in which the Asn residue is mutated to Asp. We also identified the deamidation or isomerization site of the lysozyme samples after incubating them under acidic or basic conditions.

Key words: asparagine deamidation, aspartate isomerization, endoproteinase Asp-N, MALDI/TOF-mass spectrometry.

Abbreviations: MALDI/TOF, matrix-assisted laser desorption/ionization time-of-flight.

Amino acid side chains or peptide backbones of proteins are susceptible to various non-enzymatic chemical reactions under physiological conditions, including deamidation, racemization, isomerization, β-elimination, exchange of disulfide bonds, and oxidation. In particular, deamidation of Asn or Gln (*1–6*, *11*, *12*), and isomerization / racemization of Asp (*3*, *7–12*) are frequently reported. These reactions are generally accelerated at higher temperatures, and they have different pH-dependencies. If such a chemical reaction occurs at the active center of an enzyme, the enzyme will be inactivated very easily. On the other hand, when various chemical modifications accumulate, a protein loses its native conformation and thereby its function (*1–2*).

Deamidation involves hydrolysis at an amide bond of Asn and Gln, and it occurs under physiological conditions (*13*). Moreover, deamidation is promoted under basic conditions and occurs more easily at Asn than at Gln. In particular, significant reaction rates have been observed under neutral pH conditions, when the Asn residue is followed by Gly in the sequence of amino acids, under which conditions Asn has a marked tendency to form the succinimide intermediate (*13–15*). Deamidation is the most probable reaction by which proteins are degraded during cell culture, purification, and preservation. Deamidation is known to lead to heterogeneity in terms of quality, and it also is known to decrease the activity of an enzyme. For example, serine hydroxymethyltransferase purified from rabbit liver cytosol with two deamidated Asn residues (Asn5 and Asn220) had lower catalytic activity than the recombinant enzyme in the nondeamidated form (*4*). The heterogeneity of purified murine monoclonal antibody MMA383 was mainly due to deamidation at Asn141 in its heavy chain and at Asn161 in its light chain (*5*). Asn107 in aged PrP was converted to Asp by a spontaneous pathway involving deamidation, resulting in an increase in the β-sheet content and a tendency to form aggregates (*6*). Such covalent variants of PrP^{C} may generate PrP^{Sc}-like species, which initiate fatal prion diseases, such as Creutzfeldt-Jacob disease.

The Asp-X peptide bond is easily isomerized or racemized via the succinimide intermediate, and L-Asp becomes β-Asp (*iso*-Asp) and D-Asp. The succinimide intermediate hydrolytically divides into a β-Asp/α-Asp mixture at an approximate ratio of 3:1. The formation of succinimide at Asp occurs most easily when Asp is followed by Gly. The optimum pH for its formation is pH 4–5. Many examples of the protein isomerization of Asp have been reported in basic fibroblast growth factor and insulin, where the main degradation products are the result of isomerization of Asp residues (*7*, *8*). Isomerization and inversion have been observed in the course of human aging. For example, the D/L-ratio of Asp residues (Asp-58, Asp-151) in αA-crystallin from human eye lenses increased with age (*9*, *10*). In the case of deamidation, Asp derived from Asn was often racemized, as was observed in tissue plasminogen activator (*11*). Site-specific deamidation and isoaspartate formation in tau proteins in the brain caused aggregation and resulted in paired helical filament formation *in vivo* (*12*).

Mass spectrometry is a powerful method to identify chemical modifications in proteins, because such modifications cause changes in the mass of protein. Mass spectrometry is often combined with peptide mapping on reversed-phase HPLC after digestion by trypsin or other enzymes, because the combination of these methods is

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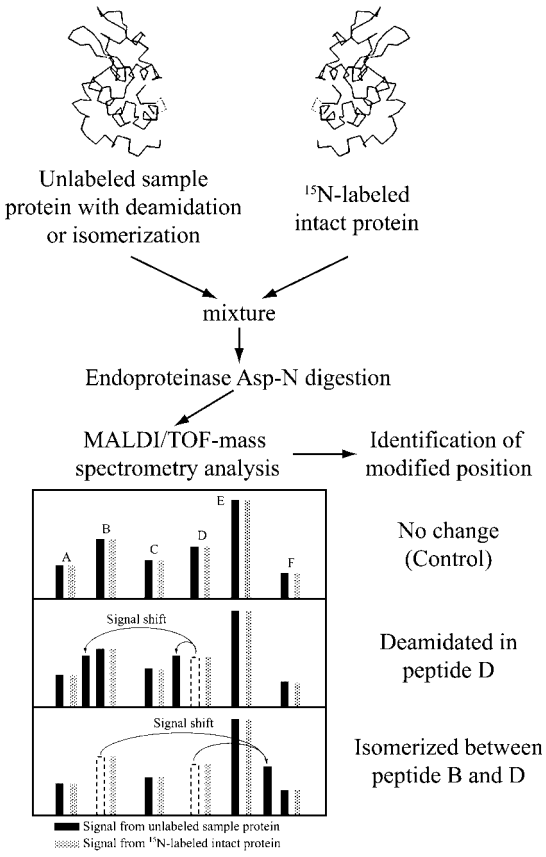

Fig. 1. **Outline for identifying position of deamidation and isomerization in sample protein, using endoproteinase Asp-N digestion, ^{15}N-labeled intact protein and MALDI/TOF-mass.**

not only highly sensitive but also enables identification of the modified position. For example, by the combination of these methods using matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) mass spectrometry, the oxidation of four methionine residues was identified in H_2O_2-treated nucleoside diphosphate kinase, because a 16 Da increase in the mass was observed in the oxidized peptide (*16*). Moreover, methionine residues that are susceptible to oxidation in recombinant human 1-antitrypsin were identified and quantified using a combination of the above methods (*17*). When mass spectromety is applied to identify deamidation, isomerization, and racemization, the subtle differences in the mass of the protein can pose a problem. Namely, the mass of protein/peptide changes by only 1 Da in the case of deamidation, and does not change in the case of either isomerization or racemization. Indeed, to detect deamidation, isomerization and racemization, the separation of peptides on reverse-phase HPLC was required in the case of lysozymes and cAMP-dependent protein kinase (*18*, *19*). Edman sequencing is useful for identifying isomerization and racemization, because these reactions stop at the isopeptide bond. Recent improvements in the accuracy and sensitivity of mass spectrometry have allowed the identification of deamidated Asn in proteins by detecting a subtle change in the mass of proteins of only 1 Da (*5*, *6*). However, achieving total accuracy by mass spectrometry would require extra experimental techniques.

In this report, we propose a convenient method for detecting deamidation of Asn and isomerization/racemization of Asp, using a combination of mass spectrometry and peptide mapping with endoproteinase Asp-N (Fig. 1). Endoproteinase Asp-N is a specific protease that cleaves the N-terminus amide bond of L-α-Asp (*20*). When Asn is deamidated to Asp in proteins or peptides, the product will be hydrolyzed by endoproteinase Asp-N and will increase the number of signals on mass spectrometry. On the other hand, when L-α-Asp in proteins or peptides is isomerized to L-β-Asp or racemized to D-Asp, the product is not hydrolyzed by endoproteinase Asp-N, and the number of signals on mass spectrometry decreases. Therefore, by comparing the number of signals and the distribution of the mass derived from MALDI/TOF analysis of the peptide mixture after endoproteinase Asp-N digestion, these chemical modifications can be selectively detected. To improve accuracy when determining the modified site, we performed mass spectrometry in the presence of the corresponding stable isotope-labeled protein (*21*). By this treatment, it becomes easy to determine the signals derived from peptide mapping with those of the corresponding labeled peptide, because we are able to obtain information concerning the difference in the mass between the sample and labeled protein signals.

In this study, we used hen egg lysozyme as a model protein, because its chemical characterization has already been investigated in detail (*1*, *3*, *18*). We tested the validity of this method by identifying the mutated site in two mutants where Asn is mutated to Asp. Furthermore, we attempted to determine the position of deamidation or isomerization, which was reported to occur in lysozyme incubated in phosphate (pH 8.0) or acetate (pH 4.0) buffer.

MATERIALS AND METHODS

Materials—Five-times-recrystallized hen egg-white lysozyme was donated by QP Company (Tokyo). Mutant lysozymes in which Asn27 and Asn44 are mutated to Asp (N27D and N44D), respectively, and ^{15}N uniformly labeled lysozyme were previously prepared in our laboratory (*22*, *23*). Resourse S (1 mL) and TSK-gel ODS-120T (4.6 × 250 mm) columns were obtained from Amersham Biosciences (Piscataway, NJ) and Tosoh (Tokyo), respectively. Endoproteinase Asp-N was purchased from Takara Bio (Otsu). All other chemicals used were of the highest quality commercially available.

Preparation of Chemically Modified Lysozymes—Deamidated or isomerized lysozyme was prepared as described below. Lysozyme solution was incubated at 40°C for 7 days in 50 mM phosphate buffer (pH 8.0) for deamidation or at 60°C for 3 days in 50 mM acetate buffer (pH 4.0) for isomerization. Each modified lysozyme was purified by cation-exchange chromatography using the Resourse S column (1 ml), which was eluted with a linear gradient of 30 ml of 0.05 M acetate buffer and 30 ml of the same buffer containing 0.5 M NaCl at pH 5, at a flow rate of 1 ml/min. The eluted solution of deteriorated lysozyme was dialyzed against purified water.

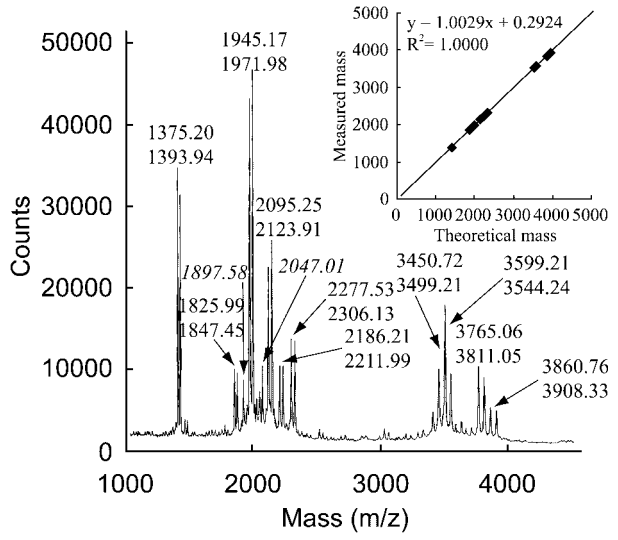

Fig. 2. **MALDI/TOF-mass spectrum of the mixture of unlabeled lysozyme and ^{15}N-labeled lysozyme after endoproteinase Asp-N digestion.** (Inset) Calibration curve of the theoretical mass from the measured mass.

Reduction and S-Carboxymethylation of Lysozymes—Fifty nanomols of hen egg-white lysozyme, mutant lysozymes (N27D and N44D), chemically modified lysozyme (deamidated and racemized), and ^{15}N-labeled lysozyme were lyophilized and dissolved in 60 μl of 0.5 M Tris-HCl buffer (pH 8.1) containing 8 M guanidine hydrochloride and 2 mM EDTA. Then 20 μl of 2-mercaptoethanol was added to each solution, and the mixture was heated at 40°C for 2 h. Then 76 mg of iodoacetic acid sodium salt was added, and the mixture was kept in the dark for 15 min. The samples were dialyzed against 10% acetic acid solution at 4°C and then lyophilized.

Proteolytic Digestion of Lysozyme Samples—Each *S*-carboxymethylated lysozyme (50 nmol) was dissolved in 50 μl of water. To each solution, the same amount of carboxymethylated ^{15}N-labeled lysozyme solution (50 μl) was added. Endoproteinase Asp-N digestion of the lysozyme mixtures (total: 100 nmol) was performed under the following conditions: a 100:1 weight ratio of substrate to enzyme was incubated in 200 μl of 200 mM ammonium bicarbonate buffer (pH 8.4) at 37°C for 15 h.

MALDI/TOF–Mass Spectrometry—Endoproteinase Asp-N–digested samples were analyzed on a Voyager Elite mass spectrometer (PerSeptive Biosystems) using α-cyano 4-hydroxy cinnamic acid as the matrix. Each peak of the mass spectrum was identified by the mass and the difference in the mass between the unlabeled sample and the corresponding ^{15}N-labeled lysozyme. Internal calibration of molecular mass was achieved by use of a standard curve derived from peptides of the ^{15}N-labeled lysozyme. The theoretical mass of the peptides generated by endoproteinase Asp-N digestion was calculated using "PeptideMass" programs (*24*).

RESULTS AND DISCUSSION

An equimolar mixture of "unlabeled" and "^{15}N-labeled" lysozymes was digested with endoproteinase Asp-N, and the mass of the digested mixture was measured by MALDI/TOF-mass spectrometry (Fig. 2). Generally, if nitrogen atoms in a protein are labeled with the ^{15}N isotope, the mass of an amino acid residue containing ^{15}N increases by 1–4 Da. The mass per amino acid residue increases by 4 Da for Arg, 3 Da for His, 2 Da for Lys, Trp, Gln and Asn, and 1 Da for the other amino acid residues. Therefore, in this study, the peptides released by digestion with endoproteinase Asp-N from unlabeled and ^{15}N-labeled lysozymes, each gave a pair of peaks on MALDI/TOF–mass spectrum, depending on its each primary structure; this phenomenon indicated the coexistence of peptides with subtle differences in the mass. We compared the masses of respective pairs of peaks from unla-

Table 1. **Expected and observed masses of peptides obtained from endoproteinase Asp-N cleavage of unlabeled and ^{15}N-labeled, carboxymethylated lysozyme, on MALDI/TOF-mass spectrometry.**

Asp-N peptide	Sequence	Expected mass (*m*/*z*)			Observed mass (*m*/*z*)		
		Hen egg lysozyme	^{15}N-labeled lysozyme	Difference	Hen egg lysozyme	^{15}N-labeled lysozyme	Difference
Peptides obtained from complete cleavage of Asp-N							
1–17	KVFGRCELAAAMKRHGL	1,945.03	1,972.03	27.00	1,945.17	1,971.98	26.81
18–47	DNYRGYSLGNWVCAAKFESNFNTQATN-RNT	3,499.57	3,544.57	45.00	3,499.21	3,544.24	45.03
48–51	DGST	379.15	383.15	4.00	–	–	–
52–65	DYGILQINSRWWCN	1,825.83	1,847.83	22.00	1,825.99	1,847.45	21.46
66–86	DGRTPGSRNLCNIPCSALLSS	2,277.06	2,306.06	29.00	2,277.53	2,306.13	28.60
87–100	DITASVNCAKKIVS	1,506.78	1,523.78	17.00	–	–	–
101–118	DGNGMNAWVAWRNRCKGT	2,093.94	2,123.94	30.00	2,095.25	2,123.91	28.66
119–129	DVQAWIRGCRL	1,374.70	1,393.70	19.00	1,375.20	1,393.94	18.74
Peptides obtained from incomplete cleavage of Asp-N							
18–51	DNYRGYSLGNWVCAAKFESNFNTQATN-RNTDGST	3,859.70	3,908.70	49.00	3,860.76	3,908.33	47.57
48–65	DGSTDYGILQINSRWWCN	2,185.96	2,211.96	26.00	2,186.21	2,211.99	25.77
66–100	DGRTPGSRNLCNIPCSALLSSDITASVN-CAKKIVS	3,764.83	3,810.83	46.00	3,765.06	3,811.05	45.99
101–129	DGNGMNAWVAWRNRCKGTD-VQAWIRGCRL	3,449.62	3,498.62	49.00	3,450.72	3,499.21	48.49

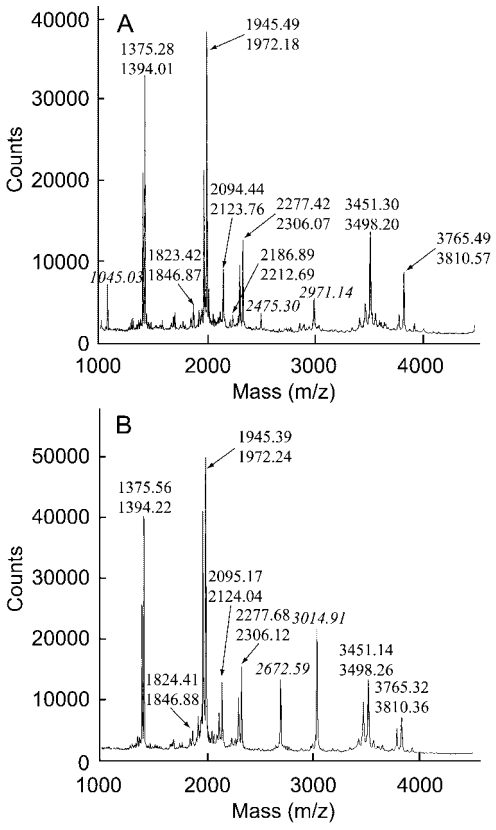

Fig. 3. **MALDI/TOF-mass spectra of the mixtures of mutant lysozyme and ^{15}N-labeled lysozyme after endoproteinase Asp-N digestion.** A, Mixture of N27D lysozyme and ^{15}N-labeled lysozyme; B, mixture of N44D lysozyme and ^{15}N-labeled lysozyme.

beled and ^{15}N-labeled peptides digested by endoproteinase Asp-N, and this information allowed the calibration of the theoretical mass (Table 1).

Although six pairs of peptides were successfully identified, peptides 48–51 and 87–100 could not be identified. Since peptide 48–51 was small, consisting of only 4 amino acid residues, it was difficult to identify using MALDI/TOF-mass spectrometry. On the other hand, peptide 87–100 consisted of 14 amino acid residues, which was a sufficient mass for detection on MALDI/TOF-mass spectrometry. To determine why this peptide could not be identified, we analyzed all peptide peaks derived from endoproteinase Asp-N digests of lysozyme, which were separated on reverse-phase HPLC, using MALDI/TOF mass spectrometry. As a result, we detected a peak with the mass corresponding to peptide 87–100 (data not shown). In general, the ionization efficiency of peptides on MALDI/TOF-mass spectrum differs according to the sequence of amino acid residues. Therefore, it was assumed that peptide 87–100 was very difficult to ionize, and could not be detected because such a large number of peptides were ionized simultaneously. Although two peaks were seen at the masses of 1,897.58 and 2,047.01 on MALDI/TOF-mass spectrometry (Fig. 2), they were considered to be derived from the self-digestion of endoproteinase Asp-N, because there was no corresponding peptide in the digest of the lysozyme by endoproteinase Asp-N.

We applied this method for the detection of a deamidated site in mutant lysozymes in which Asn is mutated to Asp. In the case of the N27D lysozyme, Asn27 is mutated to Asp; three additional peaks appeared at the masses of 1,045.03, 2,475.30, and 2,971.14, after the correction of masses using the peptides of the ^{15}N-labeled

Table 2. **Assignment of peaks on MALDI/TOF-mass spectrometry derived from the digests of mutant lysozyme with endoproteinase Asp-N.** The correct masses were achieved with the calibration curve of peptides derived from the ^{15}N-labeled lysozyme.

(A) N27D

Disappeared peptide

Asp-N peptide	Sequence	Expected mass (m/z)			Observed mass (m/z)		
		Hen egg lysozyme	^{15}N-labeled lysozyme	Difference	N27D	^{15}N-labeled lysozyme	Difference
18–47	DNYRGYSLGNWVCAAKFESNFNTQATNRNT	3,499.57	3,544.57	45.00	–	3,545.01	–

Appeared peptides

Observed mass (m/z)	Corresponding peptide	
	Assigned sequence	Expected mass (m/z)
1,045.03	DNYRGYSLG	1,044.47
2,475.30	DWVCAAKFESNFNTQATNRNT	2,475.10
2,971.14	KVFGRCELAAAMKRHGLDNYRGYSLG	2,970.48

(B) N44D

Disappeared peptide

Asp-N peptide	Sequence	Expected mass (m/z)			Observed mass (m/z)		
		Hen egg lysozyme	^{15}N-labeled lysozyme	Difference	N44D	^{15}N-labeled lysozyme	Difference
18–47	DNYRGYSLGNWVCAAKFESNFNTQATNRNT	3,499.57	3,544.57	45.00	–	3,545.06	–

Appeared peptides

Observed mass (m/z)	Corresponding peptide	
	Assigned sequence	Expected mass (m/z)
2,672.59	DRNTDGSTDYGILQINSRWWCN	2,672.18
3,014.91	DNYRGYSLGNWVCAAKFESNFNTQAT	3,014.34

Table 3. **Assignment of peaks on MALDI/TOF-mass spectrometry derived from the digests of chemically modified lysozyme with endoproteinase Asp-N.** The correct masses were achieved with the calibration curve of peptides derived from the ^{15}N-labeled lysozyme.

(A) Incubation in 50 mM phosphate buffer (pH 8.0)

Disappeared peptide

Asp-N peptide	Sequence	Expected mass (m/z)			Observed mass (m/z)		
		Hen egg lysozyme	^{15}N-labeled lysozyme	Difference	Hen egg lysozyme	^{15}N-labeled lysozyme	Difference
101–118	DGNGMNAWVAWRNRCKGT	2,093.94	2,123.94	30.00	–	2,122.23	–

Appeared peptide

Observed mass (m/z)	Corresponding peptide	
	Assigned sequence	Expected mass (m/z)
1,924.79	DGMNAWVAWRNRCKGT	1,922.88

(B) Incubation in 50 mM acetate buffer (pH 4.0)

Disappeared peptide

Asp-N peptide	Sequence	Expected mass (m/z)			Observed mass (m/z)		
		Hen egg lysozyme	^{15}N-labeled lysozyme	Difference	Hen egg lysozyme	^{15}N-labeled lysozyme	Difference
101–118	DGNGMNAWVAWRNRCKGT	2093.94	2123.94	30.00	–	2123.39	–

Appeared peptide

Observed mass (m/z)	Corresponding peptide	
	Assigned sequence	Expected mass (m/z)
3,582.85	DITASVNCAKKIVSDGNGMNAWVAWRN-RCKGT	3,581.71

lysozyme (Fig. 3A). Because, Asn27 changes to Asp in this mutant, peptide 18–47 in N27D lysozyme was divided into two peptides, *i.e.*, 18–26 and 27–47, after digestion. The theoretical masses of these two peptides were 1,044.47 and 2,475.10, respectively. Therefore, these additional peaks (1,045.03, 2,475.30, and 2,971.14) were assigned to peptides 18–26, 27–47, and 1–26, respectively (Table 2A). This result suggests that endoproteinase Asp-N was cleaved at the N-terminus of the 27th residue in the N27D lysozyme. On the other hand, in the case of the N44D lysozyme, Asn44 is mutated to Asp; two additional peaks appeared at the masses of 2672.59 and 3014.91, after the correction of masses using the peptides of the ^{15}N-labeled lysozyme (Fig. 3B). Because Asn44 changes to Asp in this mutant, the peptide 18–47 in N44D was divided into two peptides, 18–43 and 44–47, after digestion, and their theoretical masses were 3,014.34 and 505.24, respectively. Therefore, we assigned these additional peaks with the masses at 2,672.59 and 3,014.91 to peptides 44–65 and 18–43, respectively (Table 2B). This result suggests that endoproteinase Asp-N was cleaved at the N-terminus of the 44th residue in the N44D lysozyme. It was concluded that the present method could be applied for the detection of the deamidation site of Asn in proteins.

The Asn and Asp residues differ in mass by only 1 Da. To detect this difference, an advanced technique and a sophisticated equipment are both required. Using the present method, it was determined that a new site of cleavage by endoproteinase Asp-N appears when the Asn residue changes to Asp. Therefore, the number and the mass of peptides observed on MALDI/TOF-mass spectrometry will change. The present method is thus useful for determining the deamidation site in a protein.

Generally, deamidation at the Asn residue in a protein occurs easily at basic pH (*1*). We obtained an unknown peak that was eluted earlier than the intact lysozyme peak on cation-exchange HPLC after incubating the lysozyme at 40°C for 7 days in 50 mM phosphate buffer, pH 8.0 (Fig. 4A). MALDI/TOF-mass spectrometry analysis of endoproteinase Asp-N digests of the unknown peak, which was isolated by cation-exchange HPLC, was performed. Fig. 5A shows that the expected peak at the mass of 2,093.94 corresponding to peptide 101–118 disappeared, and that new peaks appeared at the masses of 1,897.29 and 1,924.79. It was found that the peak at the mass of 1,924.79 was equivalent to the position of 103–118, as determined by fitting the primary structure of the lysozyme to the mass of this peak (Table 3A). This suggested that peptide 101–118 was cleaved by endoproteinase Asp-N at the N-terminus of the 103rd residue in the lysozyme, because Asn103 changed to Asp during incubation. In addition, the peak at the mass of 1,897.29 may be considered as the product resulting from self-digestion of endoproteinase Asp-N.

On the other hand, isomerization at the Asp residue in a protein is reported to occur at acidic pH (*3*). We obtained an unknown peak that was eluted earlier than the unchanged lysozyme peak on cation-exchange HPLC after incubating the lysozyme at 60°C for 3 days in 50 mM acetate buffer, pH 4.0 (Fig. 4B). MALDI/TOF-mass spectrometry analysis of endoproteinase Asp-N digests of the unknown peak was performed. Fig. 5B shows that the expected peak at the mass of 2,093.94 corresponding to peptide 101–118 disappeared, and that the new peak appeared at the mass of 3,582.85, which corresponded to peptide 87–118, as determined by fitting the primary structure of the lysozyme to the mass of this peak (Table 3B). The results indicate that a chemical modification occurred at Asp101 in the lysozyme, and that peptide 101–118 could not be cleaved by endoproteinase Asp-N. Therefore, we concluded that Asp101 in the lysozyme had isomerized during incubation.

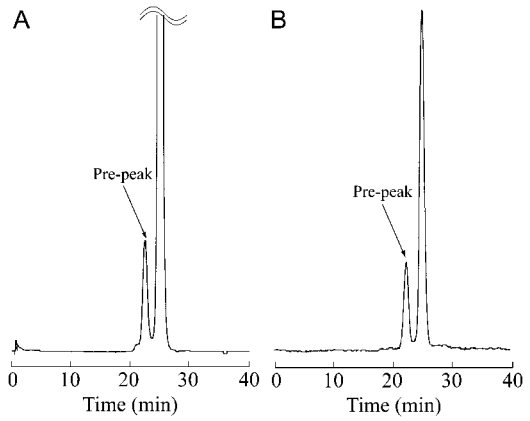

Fig. 4. **Cation-exchange HPLC of the incubated lysozyme on Resourse S (1 ml) column.** The column was eluted with a linear gradient of 30 ml of 0.05 M acetate buffer and 30 ml of the same buffer containing 0.5 M NaCl at pH 5, at the flow rate of 1 ml/min. A, After incubation at 40°C for 7 days in 50 mM phosphate buffer (pH 8.0); B, after incubation at 60°C for 3 days in 50 mM acetate buffer (pH 4.0).

As described above, the present method made it possible to identify the position of the deamidation of the Asn residue or the isomerization of the Asp residue in the lysozyme. Therefore, this method has proven useful for the identification of the deamidation of the Asn residue and the isomerization of the Asp residue, which has been difficult to determine by conventional methods. However, when the Asp residue produced by the deamidation of the Asn residue is also isomerized, it remains difficult to detect the change by this method. In this case, Edman degradation would be useful, as it analyzes the peptide sequence (*25*). On the other hand, ^{15}N-labeled protein was used in this method as a means to identify the signals and calibrate the mass of signals. The complexity of the signals in the MALDI/TOF-mass spectrum is, however, a problem in the application of this method to proteins of high molecular weight. Therefore, it is difficult to identify each signal to corresponding theoretical peptides. Moreover, the application of ^{15}N-labeled protein is limited to proteins that have been cloned and expressed. However, these problems are solvable by calibrating the mass using the standard peptides with known molecular weights.

This paper introduces a novel method to identify the site of deamidation of the Asn residue or the isomerization of the Asp residue in hen egg-white lysozyme, which was used as a model protein. In the case of a protein of low molecular weight, such as a lysozyme, the identification of modified site is possible using peptide mapping of endoproteinase Asp-N digestion on reverse-phase HPLC, without using the present method. However, in the case of proteins of high molecular weight, it is generally difficult to perform peptide mapping of good quality, and therefore the present method may be beneficial for identifying modified sites. Moreover, it is expected that the application of liquid chromatography and mass spectrometry combined with this method will improve the capability of detection (*26*). Although charge or size heterogeneity of proteins is often detected by electrophoresis

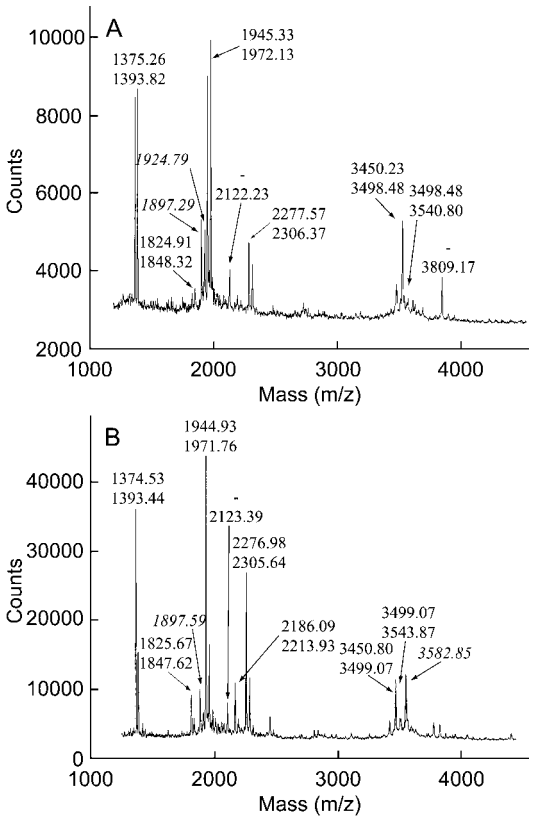

Fig. 5. **MALDI/TOF-mass spectra of the mixtures of chemically modified lysozyme and ^{15}N-labeled lysozyme after endoproteinase Asp-N digestion.** A, Mixture of the pre-peak in Fig. 4A and ^{15}N-labeled lysozyme; B, mixture of the pre-peak in Fig. 4B and ^{15}N-labeled lysozyme.

(*e.g.*, by isoelectric focusing or SDS-PAGE), this method may be useful for the identification of chemical modifications in these heterogeneous proteins by combining in-gel digestion methods that have been recently reported (*27*).

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