Original Paper

Establishment of a Two-Dimensional HPLC-MS/MS Method Combined with DCI/D₂O Hydrolysis for the Determination of Trace Amounts of D-Amino Acid Residues in Proteins

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Abstract

A two-dimensional (2D) HPLC-MS/MS method has been established for the determination of trace amounts of D-amino acid residues in proteins. D-Amino acid residues are now increasingly recognized as biomarkers of diseases and key moieties for the regulation of protein structures/functions, therefore, a sensitive analytical method is highly required. However, non-negligible amounts of D-amino acids are produced by chemical racemization during the hydrolysis of proteins, and a sensitive determination of D-amino acid residues is normally difficult. In the present study, DCI/D₂O hydrolysis is adopted and the produced deuterated D-amino acids are distinguished from naturally-occurring D-amino acids in the proteins using the 2D-HPLC-MS/MS system. D-Ala, D-Asp, D-Glu, D-Pro and D-Ser (frequently observed D-amino acids in higher animals) were selected as the targets, and the sensitive determination (around 1% or less of the L-forms) could be carried out. As an application, the D-amino acid residues in ovalbumin (OVA) were determined, and the presence of a significant amount of D-Ser (1.8% of L-Ser) was demonstrated.

Keywords: Enantiomer separation; D-Amino acid residue; 2D-HPLC-MS/MS

1. Introduction

Amino acids are the major constituents of living organisms, and widely present in their free forms and protein/peptide bound forms. All proteinogenic amino acids, except glycine, have optical isomers (enantiomers), namely the D- and L-forms, and L-amino acids are predominant in the higher animals. However, from the 1980s, several D-amino acids have been reported to be present as their free forms in mammalian tissues including human beings, and these D-amino acids are expected to be novel physiologically active substances and biomarkers

[1-5]. In the proteins and peptides, D-amino acid residues, especially D-Asp, have also been reported in various disease/aged proteins including Alzheimer's disease and cataract [6-12]. Because Asp residues can be easily transformed into succinimidyl intermediates, the chiral inversion from L-Asp to D-Asp frequently occurs, therefore, D-Asp residues are often observed in disease/aged proteins [6]. However, some other D-amino acid residues, such as D-Ser and D-Asn, were also formed in a few protein samples [13-15]. These reports suggest that various D-amino acid residues are likely to occur without the

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formation of succinimidyl intermediates. Because these D-amino acid residues are expected to be biomarkers of disease/aging, and also expected to be the key to regulate protein functions, a comprehensive analytical method for the determination of trace amounts of D-amino acid residues is highly required.

Currently, several methods have already been reported. Normally, the proteins were enzymatically digested, and the peptides containing the target D-amino acid residues are determined as diastereomers [16-17]. Edman degradation combined with enantioselective analysis of the produced PTH amino acids has also been reported [18]. However, these methods are complicated and applicable to limited protein/peptide samples having specific target sequences. For the “non-targeted” determination of trace amounts of D-amino acid residues, the proteins/peptides must be hydrolyzed before analysis. Therefore, the racemization of amino acids during hydrolysis is a serious problem (non-negligible amounts of the D-amino acids are produced by the chemical racemization). In some cases, an extrapolation method was used to evaluate the amounts of D-amino acids naturally occurring in protein/peptide samples by analyzing the time course of the D-amino acid formation [19]. However, the racemization of amino acids during hydrolysis was not linear and not applicable to the trace analysis.

To avoid the interference by “chemically racemized” amino acids during hydrolysis, the DCI/D₂O (deuterium chloride in deuterium oxide) hydrolysis method has been established. Peptide or protein samples are hydrolyzed using DCI/D₂O, resulting in the formation of “deuterated” amino acids at their α position during the chemical racemization [20-21]. Therefore, naturally-occurring D-amino acids could be distinguished using a mass spectrometer from those generated by chemical racemization during hydrolysis (Fig. 1). This method is a straightforward approach, however, the target amino acids were still limited, and expansion to include all frequently-observed D-amino acids in higher animals is highly required.

In the present study, an analytical method has been established for the determination of D-Ala, D-Asp, D-Glu, D-Pro and D-Ser (these 5 D-amino acids are frequently observed in vertebrates [2-5]) residues in protein samples. For the analysis, a two-dimensional HPLC-MS/MS system was adopted after the DCI/D₂O hydrolysis. The method was evaluated using the standard L-amino acids, and applied to a real protein sample (ovalbumin, OVA).

2. Experimental

2.1. Materials

Acetonitrile (MeCN) of HPLC grade was obtained from Nacalai Tesque (Kyoto, Japan). DL-Pro was obtained from

Fig. 1. A graphical image of DCI/D₂O hydrolysis. Only the D-amino acids produced during hydrolysis are deuterated at the α-hydrogen. Therefore, the naturally-occurring D-amino acids (not deuterated) could be distinguished from those produced during hydrolysis.

Kanto Chemical (Tokyo, Japan). DL-Asp, L-Asp, DL-Ser and L-Ser were purchased from Nacalai Tesque. DL-Glu was from Tokyo Chemical Industry (Tokyo, Japan) and DL-Ala, L-Ala, L-Glu and L-Pro were from Wako Pure Chemical Industries (Osaka, Japan). Deuterium chloride (20% DCI in D₂O) and deuterium oxide (D₂O) were purchased from Acros Organics (Geel, Belgium). 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was obtained from Tokyo Chemical Industry. Methanol (MeOH) of HPLC grade, trifluoroacetic acid (TFA) and boric acid were purchased from Wako. Ovalbumin (OVA, purified) was obtained from the Worthington Biochemical Corporation (Lakewood, NJ, USA). Water was purified using a Milli-Q gradient A 10 system (Merck Millipore, Billerica, MA, USA). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Sample preparation

Standard L-amino acids (0.5 μmol) or ovalbumin (50 μg) in water were dried under reduced pressure, then heated at 110°C for 20 h with the vapor of 6 M DCI/D₂O. The residue was dissolved in 100 μL of water, and an aliquot (10 μL) was placed in a brown tube (MCT-060-X, Axygen, Union city, CA, USA). To the tube, 10 μL of 400 mM sodium borate buffer (pH 8.0) and 1 μL of 200 mM NBD-F in anhydrous MeCN were added, then heated at 60°C for 2 min. After heating, 79 μL of an aqueous 0.5% (v/v) TFA was added, and 10 μL of the reaction mixture was subjected to the two-dimensional HPLC system described in section 2.3.

2.3. Two-dimensional HPLC determination of D- and L-amino acids

The HPLC system (Nanospace SI-2 series, Shiseido, Tokyo, Japan) for the first dimension consisted of two pumps (3201 and 3101), an auto sampler (3023), a column

oven (3004) and a fluorescence detector (3013). The HPLC system for the second dimension consisted of a degasser (3202), two pumps (3201 and 3301), an auto sampler (3033), a column oven (3014), a fluorescence detector (3213) and a tandem mass spectrometer (API-5000 or Triple Quad™ 5500, AB Sciex, Framingham, MA, USA). The target amino acids (Ala, Asp, Glu, Pro, Ser) were separated by a capillary monolithic ODS column (0.53 mm i.d. x 1000 mm, 45°C, prepared in a fused silica capillary, provided by Shiseido) in the first dimension as their NBD derivatives by gradient elution using aqueous mobile phases containing MeCN and TFA. The fractions of the NBD-amino acids were collected and introduced into the second dimension. In the second dimension, the NBD-D- and L-amino acids were separated using Pirkle-type narrowbore-enantioselective columns, namely, KSAACSP-001S (1.5 mm i.d. x 250 mm, 25°C, self-packed, materials were obtained from Shiseido) and Sumichiral OA-3200S (1.5 mm i.d. x 250 mm, 25°C, self-packed, materials were obtained from Sumika Chemical Analysis Service, Osaka, Japan) with the mixed solution of MeOH-MeCN containing formic acid. The fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm. These NBD-D- and L-amino acids were also determined by an ESI-tandem mass spectrometer. The MS/MS conditions are listed in Table 1.

Table 1. MS/MS conditions for NBD-Ala, Asp, Glu, Pro and Ser enantiomers.

MS/MS condition	Amino acids (Q1 / Q3)				
	Ala (253/236)	Asp (299/175)	Glu (313/249)	Pro (279/262)	Ser (269/206)
CUR	20	20	20	20	20
GS1	40	40	40	30	40
GS2	40	80	60	70	60
IS	5500	5500	5500	5500	5500
TEM	650	600	400	700	600
CAD	10	10	6	11	12
DP	121	116	131	95	100
EP	10	10	10	10	10
CE	21	37	21	21	31
CXP	20	20	20	18	24

CUR: Curtain Gas (psi), GS1: Ion Source Gas 1 (psi), GS2: Ion Source Gas 2 (psi), IS: Ionspray Voltage (V), TEM: Temperature (°C), CAD: Collision Gas (psi), DP: Declustering Potential (V), EP: Entrance Potential (V), CE: Collision Energy (eV), CXP: Collision Cell Exit Potential (V).

3. Results and discussion

3.1. Establishment of an enantioselective two-dimensional HPLC-MS/MS system

For the determination of the D-Ala, D-Asp, D-Glu, D-Pro and D-Ser residues in the proteins, an enantioselective two-dimensional HPLC system combining a monolithic ODS column and enantioselective columns has been established. The five target D-amino acids were selected because these D-amino acids are frequently observed in higher animals (as their free forms) [2-5]. In the first dimension, the five target NBD-amino acids were separated

using a capillary monolithic ODS column, and the elution conditions were optimized. As a result, the five target NBD-amino acids were separated within 90 min as D plus L mixtures, and isolated from other amino acids by gradient elution using 5-18% (v/v) MeCN containing 0.05% (v/v) TFA. The flow rate of 25 µL/min was selected due to the appropriate column pressure.

The isolated NBD-amino acid fractions were introduced into the enantioselective columns to separate and determine the enantiomers in the second dimension. Concerning the stationary phases, a Sumichiral OA-3200S column was used to separate the Ala, Asp, Glu and Ser enantiomers. On the other hand, a KSAACSP-001S column was used to separate the Pro enantiomers, and the mobile phase conditions were investigated. As a result, the enantiomers of NBD-Ala, Glu and Ser were sufficiently separated ($\alpha=1.4$ for Ala, 1.3 for Glu, 1.4 for Ser) within 20 min using 0.8% formic acid in a mixed solution of MeOH-MeCN (20:80, v/v). Concerning NBD-Asp, a good enantiomer separation ($\alpha=1.3$) was obtained within 20 min using 2% formic acid in a mixed solution of MeOH-MeCN (50:50, v/v). The enantiomers of Pro were successfully separated ($\alpha=1.2$) within 15 min using 0.8% formic acid in a mixed solution of MeOH-MeCN (20:80, v/v). The flow rate was 150 µL/min. The peaks of NBD-amino acid enantiomers were monitored by fluorescence detectors in the first and second dimensions, and also monitored by an ESI-tandem mass spectrometer in the second dimension. The MS/MS conditions for the determination of the target NBD-amino acids were optimized by the direct infusion of NBD-amino acids into the MS/MS instrument. The typical chromatograms of the NBD derivatives of standard DL-Ala, Asp, Glu, Pro and Ser are shown in Fig. 2.

Fig. 2. Chromatograms of standard D- and L-Ala, Asp, Glu, Pro and Ser. The amino acid enantiomers were separated as their scalemic mixtures by a monolithic ODS column in the first dimension (A). The isolated NBD-D- and L-amino acids were separated by the enantioselective columns in the second dimension (B). The detailed HPLC conditions are described in the text.

The present method combining DCI/D₂O hydrolysis and two-dimensional HPLC-MS/MS was evaluated in the first step by analyzing standard L-amino acids. The five target L-amino acids (100 nmol) were heated at 110°C for 20 h with the vapor of DCI/D₂O, and the ratios of the D-forms to total amino acids (%D, D/(D+L)×100) were determined by both the fluorescence detector and the MS/MS. Before hydrolysis, the D-forms were not observed for all the standard L-amino acids. However, after hydrolysis, the values of %D obtained by the fluorescence detection were 1.3% for Ala, 4.2% for Asp, 2.2% for Glu, 2.7% for Pro and 0.1% for Ser. On the other hand, the %D values acquired by MS/MS were clearly lower than those obtained by the fluorescence detector; the values being 0.2% for Ala, 0.9% for Asp, 1.0% for Glu, 0.3% for Pro and 0.1% for Ser. These results indicated that non-negligible amounts of the D-amino acids were produced during the DCI/D₂O hydrolysis, and these deuterated D-amino acids could be easily distinguished from the naturally-occurring (non-deuterated) D-amino acids by MS/MS.

Until now, several methods have been reported for the determination of D-amino acid residues in peptides and/or proteins. Traditionally, the target proteins were enzymatically degraded, and the produced peptides containing the D-amino acid residues were thoroughly checked as diastereomers by reversed-phase HPLC [16]. An enantioselective sequencing method combining Edman degradation and enantioselective analysis of the produced PTH amino acids has also been reported [18]. For the screening of diastereomeric peptides containing D-amino acids, the use of an LC-MS/MS method is also effective [17]. However, these analytical methods are complicated and only applicable to selected sequences of peptides mainly containing Asp residues. An extrapolation method analyzing the time course of the D-amino acid formation and estimating the presence of the D-forms at “0” time (naturally-occurring D-amino acids) has also been used to evaluate the racemization of the amino acid residues in the proteins and peptides [19]. However, the generation of the D-amino acids during hydrolysis was not linear and not suitable in most cases.

In 1981, Liardon *et al.* established a DCI/D₂O hydrolysis GC-MS method to analyze the presence of D-amino acids in proteins and peptides [20]. By using the DCI/D₂O hydrolysis, the D-amino acids generated during hydrolysis are deuterated and easily distinguished from non-deuterated D-amino acids by MS. However, the derivatizing conditions for the GC analysis are severe and complicated, and not applicable to all amino acids. Miyamoto *et al.* developed a DCI/D₂O hydrolysis method using LC-MS/MS, and several trace amounts of D-amino acid residues were determined using this method (limit of detection of D-form is around 1% of the L-form) [21]. However, the target amino acids are still limited, and designing an improved method for D-amino acids containing all frequently observed ones in higher animals is highly required. In the present study, the DCI/D₂O hydrolysis method was combined with an enantioselective two-dimensional HPLC-MS/MS system. Using this method, 5 amino acid enantiomers (Ala, Asp, Glu, Pro, Ser, frequently observed in higher animals) could be simultaneously analyzed. The present method would be useful to determine trace amounts of D-amino acid residues, and applications to protein/peptide samples are expected.

3.2. Determination of D-amino acid residues in ovalbumin

As an application of the present method to a real protein sample, OVA was analyzed using the 2D-HPLC-MS/MS system described in section 3.1. The OVA sample was hydrolyzed with the vapor of 6 M DCI/D₂O and the produced amino acids were analyzed after derivatization with NBD-F. As a result, the %D values obtained using the fluorescence detector were 2.4% for Ala, 5.1% for Asp, 4.2% for Glu, 3.0% for Pro and 1.8% for Ser. On the other hand, the %D values obtained using the 2D-HPLC-MS/MS method were 0.4% for Ala, 1.2% for Asp, 1.7% for Glu, 0.3% for Pro and 1.8% for Ser. The chromatograms obtained in the second dimension are shown in Fig. 3. The %D values obtained by the fluorescence detector were significantly higher than those obtained by MS/MS, again indicating that the deuterated D-amino acids produced during DCI/D₂O hydrolysis were easily distinguished by

Fig. 3. Chromatograms of D- and L-Ala, Asp, Glu, Pro and Ser in OVA obtained in the second dimension. (a) The NBD-amino acid enantiomers were detected by a fluorescence detector. (b) The NBD-amino acids were detected using an MS/MS. The detailed HPLC conditions were described in the text.

MS/MS even using a real protein sample. In OVA, %D values of Ala, Asp, Glu and Pro, observed by MS/MS were almost the same as or slightly higher than those obtained for the standard L-amino acids after the same DCI/D₂O treatment. On the other hand, the %D-Ser observed in OVA was clearly higher than that for standard L-Ser. These results strongly indicated that some D-Ser residues naturally occurred in the OVA.

Currently, several D-amino acid residues were observed in proteins. D-Asp residues were detected in various protein samples, such as the β -amyloid protein of Alzheimer's disease and the crystallin of cataract [6-12]. Therefore, the D-Asp residues are the focus of attention as novel biomarkers and therapeutic targets. On the other hand, the β -amyloid protein of Alzheimer's disease has been reported to contain D-Ser residues [13]. Concerning OVA, S-OVA, the thermal stabilizing form of OVA, is produced during the storage of eggs. Yamasaki *et al.* reported that Ser-164, Ser-236, Ser-320 were changed to the D-form in the S-OVA [14]. The estimated %D is 7.9%. In the present study, %D-Ser was determined to be 1.8% in the commercially-available OVA stored in a freezer. These results suggest that L-Ser in the OVA could be converted to D-Ser during the storage of purified OVA, or naturally occurring in the native OVA. The present results also suggest that L-amino acids in various proteins may be transformed to D-amino acids simply by storage in a freezer, and the spontaneous generations of D-amino acids could be determined using the present method. Therefore, the DCI/D₂O hydrolysis and two-dimensional HPLC MS/MS method established in the present study would be useful to determine trace amounts of D-amino acid residues in protein samples.

4. Conclusion

In the present investigation, a sensitive analytical method combining a 2D-HPLC-MS/MS system and DCI/D₂O hydrolysis has been established for the determination of D-amino acid residues in proteins. Using the present method, trace amounts of D-amino acid residues (around 1% of the L-amino acids) could be determined. In the commercially-available OVA, a non-negligible amount of D-Ser was observed. These results indicated that the D-amino acid residues are naturally present in commercially-available proteins, or might be formed during storage. These D-amino acid residues are also likely to be present in various proteins in higher animals, and further studies using disease/aged proteins are now in progress.

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