C146-E420

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Technical Report

A new method for the analysis of increased D-amino acids in proteins due to aging

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Abstract:

In recent years, it has become clear that the quantity of D-aspartic acid (Asp) in proteins of aging tissues such as teeth, bones, lenses, arterial walls, ligaments, brain, and skin increases with age and is related to the onset of age-related diseases such as cataracts, arteriosclerosis, Alzheimer's disease, multiple sclerosis, and scleroderma. Although the mass of the peptide containing Asp isomerized from the L-isomer to the D-isomer is the same as that of the original peptide, an MRM measurement using LC-MS/MS can detect peaks with a different retention time on the LC, and it is expected that the isomerization ratio of the amino acid in the peptide will be utilized as a molecular index of aging. This article describes a highly-sensitive analytical method for quantitation of D-amino acids in proteins using Microflow LC-MS/MS.

Keywords: D-amino acids in peptides, Aging, Microflow LC-MS/MS

1. Introduction

Proteins are polymers of amino acids. Amino acids have enantiomers consisting of L-(laevo) and D-(dextro) isomers, whose physical and chemical properties are same. Chemical synthesis of the amino acids produces a mixture the isomers in equal quantities. However, since living things can synthesize only L-amino acids, D-amino acids were believed not to be found in nature. But it has recently become clear that free D-amino acids are present in peptides and proteins. It has also been shown that the free D-amino acids include D-serine (D-Ser) and D-aspartic acid (D-Asp) in mammalian brains and testes, and these play important roles in neurotransmission and cell differentiation. In addition, there are enzymes involved in the metabolism and synthesis of D-Ser and D-Asp which control their levels in the body. Thus, the functions and physiological significance of the D-amino acids in the L-amino acid world have been elucidated one after another. Since the maintenance of a one-handed structure consisting of only L-amino acids in the proteins is important for the folded form and function of the proteins, it was thought that proteinogenic amino acids do not flip from L- to D- isomers over the course of a lifetime. Recently, however, D-Asp has been found to increase with age in tissues such as teeth, bones, lenses, arterial walls, ligaments, brain, and skin, and has been associated with cataracts, age-related macular degeneration, arteriosclerosis, and Alzheimer's disease. The D-amino acids are generated by racemization from the corresponding L-amino acids. Among 19 constituent amino acids with enantiomers, Asp, which has a high racemization rate, isomerizes to the D-isomer, and is found in many aging tissues[1]. The D-Amino acids in the proteins are thought to be a molecular index of aging.

2. Analysis of D-amino acids in proteins

2-1. The diastereomer method

Because L-amino acids and D-amino acids have identical physical and chemical properties, L- and D-isomers cannot be distinguished by conventional amino acid analysis. Therefore, for analysis of D- and L-amino acids, chiral columns are used to introduce D- and L-amino acids into a liquid or gas chromatograph, else D- and L-amino acids are converted into diastereomeric derivatives and analyzed using conventional reversed-phase columns. For analysis of D-amino acids in peptides and proteins, samples are hydrolyzed to convert to free amino acids and analyzed by the method described above. However, even if proteins, to which several tens to hundreds of amino acids are attached, are hydrolyzed and derivatized, and the D-amino acids are analyzed by the same method as described above, only the average racemization rate of multiple amino acid residues for each amino acid can be obtained. In order to determine which of the amino acids in the protein are racemized, the protein is isolated and purified, then treated with an enzyme such as trypsin to fragment into peptides. After the peptides are separated by reversed-phase liquid chromatography (RP-HPLC) and fractionated, they are identified and hydrolyzed. Resulting free amino acids are converted into the diastereomeric derivatives described above and D-amino acid analysis is performed[2].

2-2. The LC-MS/MS method

The method described in 2-1 has the drawbacks of requiring complex processes, a long analysis time and a large sample volume. An analytical method using a liquid chromatograph mass spectrometer (LC-MS/MS) has been developed in response to this problem. As a specific example, identification of the isomerization sites of Asp residues in proteins in the lens of the eye is described below. In this method, proteins in the lens were isolated and homogenized, and

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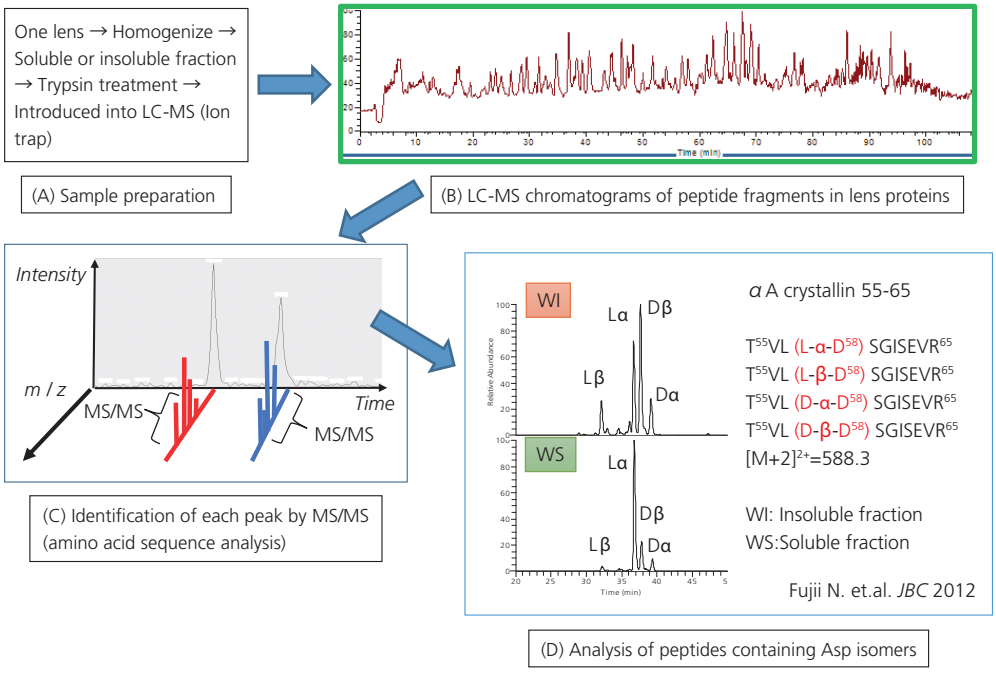

Fig. 1 New Asp isomer search and identification method using LC-MS/MS

separated into soluble (WS) and insoluble (WI) fractions by centrifugation. After trypsinization of the respective fractions, resulting peptide fragments were analyzed by LC-MS/MS (Figure 1 A-C.). LC-MS/MS is commonly used for shotgun proteomics because it can separate tryptic digests of proteins, identify peptides, and analyze the sites of modifications such as oxidation, deamidation and phosphorylation. Peptides that have undergone oxidation, deamidation, or phosphorylation differ in mass from the original peptide and are therefore suitable for post-translational modification analysis. However, there is no difference in the mass of peptides containing amino acid isomers, so even if D-amino acids are present in the peptides, they cannot be distinguished from the peptides containing L-amino acids. Nevertheless, it was found that the peptides containing D-amino acids were eluted at a different time from those containing L-amino acids. Applying this method reveals that isomer-containing peptides have the same mass and sequence and are separated into multiple peaks. As an example, Fig. 1 (D) shows a mass chromatogram of a peptide $T^{55}VLD^{58}SGISEVR^{65}$ ($[M + 2H]^{2+}$ = 588.3) containing Asp-58 in αA-crystallin in the lens of the eye.

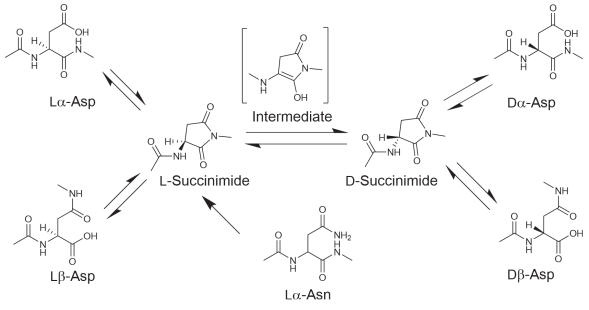

Fig. 2 Isomerization mechanism for Asp/Asn residues in proteins

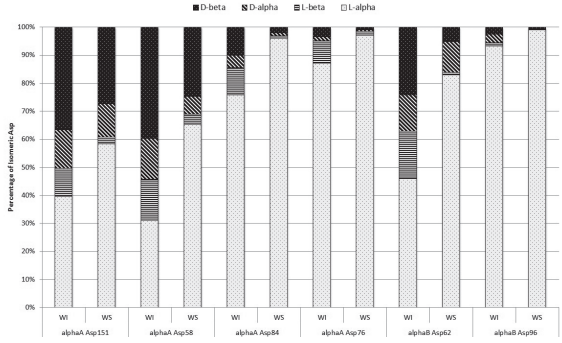

Fig. 3 Isomerization rates of Asp 58, 76, 84, and 151 residues in αA-Crystallin and Asp 62 and 96 residues in αB-Crystallin in soluble (WS) and insoluble (WI) fractions of the lens of a person in their 80s
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Normally, only one peak should be obtained for a peptide with only one mass isomer, but in this peptide, as shown in Fig. 2, Asp 58 residues were inverted and isomerized from normal Lα-Asp residues through a 5-membered cyclic imide to Lβ-, Dα-, and Dα-Asp residues, resulting in 4 peaks. The order of elution on the LC was $T^{55}VLD^{58}$(Lβ)SGISEVR^{65}, $T^{55}VLD^{58}$(Lα)SGISEVR^{65}, $T^{55}VLD^{58}$(Dβ)SGISEVR^{65}, $T^{55}VLD^{58}$(Dα)SGISEVR^{65}. Their identification was determined by chemical synthesis of each peptide, matching with elution time[3], and combination with an enzymatic method for recognizing Asp isomers[4]. The relative ratios of the four Asp isomers were calculated from the area ratios of the mass chromatograms. This method revealed that residues Asp -58, 76, 84, and -151 were formed in αA-crystallin, and residues Asp -36, 62, and 96 were formed in αB-crystallin (Fig. 3). In addition, αA- and αB-crystallins are originally present in the WS fraction, but they aggregate and migrate to the WI fraction with age. As shown in Fig. 3, the isomerization rate increased in the WI fraction for all Asp residues, suggesting that isomerization of the Asp residues is involved in protein aggregation and insolubilization[3].

2-3. Automated analysis of multiple D-amino acids with MRM

Using the method described in 2-2, the determination of the Asp isomer in crystallin and the ratio of the 4 isomers was completed in a significantly shorter analysis time than the diastereomer method described in 2-1. The shotgun method described in 2-2 is useful for the analysis of proteins with high expression levels. Since α-crystallin is a highly expressed protein in the lens, the Asp isomer site could be determined and the isomer ratio in α-crystallin calculated with the method described in 2-2. However, the shotgun method is inefficient when many samples and isomerization sites are analyzed. Since the Asp isomerization sites in α-crystallin could be limited in the shotgun method, the next step is to determine the isomer ratio of Asp efficiently by automatically analyzing a large number of samples. Therefore, an analysis was performed using MRM (Multiple Reaction Monitoring) method on a triple quadrupole mass spectrometer. MRM has traditionally been used for quantitative analysis of small molecules, but recently it is also used for proteomics analysis.

MRM detects specific peptides as targets. First, ionized peptides eluted from the LC are introduced into the MS, and some peptide ions are selected to be measured using the first mass filter Q1. The resulting mixture of peptides passes to Q2 where the peptides are cleaved. In Q3, peptides with specific ion masses are selected from these fragmented ions and pass to the detector (Fig. 4a). In the MRM method, combining the specific peptide mass with the fragment ion mass (MRM transition) makes it possible to obtain a chromatogram with a high S/N ratio. Here the equipment was a Nexera Mikros LC, which supports micro flow rates, and a triple quadrupole mass spectrometer LCMS-8060, both from Shimadzu (Fig. 7). This resulted in 10 times higher sensitivity than that of a conventional LCMS system. Fig. 4b shows results for a typical peptide with no isomers, with only one peak. Fig. 4c shows the results for a peptide with four Asp isomers, where four peaks were obtained. A specific example is the simultaneous analysis of Asp isomers in human lens α A-crystallin using the MRM method. In the MRM method, the MRM transitions need to be optimized before the analysis. Treatment of human αA-crystallin with trypsin produces 21 peptide fragments (Fig. 5). Each time the MRM transition was optimized for the next Asp-containing peptide out of all the peptide fragments.

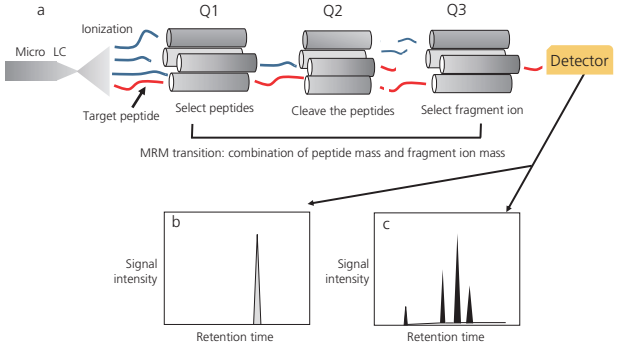

Fig. 4 Coupled analysis of Asp isomers using Multiple Reaction Monitoring (MRM)

1 MDVTIQHPWFK | R | TLGPFYPSR | LFDQFFGEGLFEYDLLPFLSSTISPYYR | Q 50
51 SLFR | TVLDSGISEVR | SDR | DK | FVIFLDVK | HF SPEDLTVK | VQDDFVEIHGK | H100
101NER | QDDHGYISR | EFHR | R | YR | LPSNVDQSALSCSLSADGMLTFCGPK | IQTGL150
151DATHAER | AIPVSR | EEK | PTSAPSS173

Fig. 5 Primary sequence of human αA-crystallin

Table 1 MRM transitions for each peptide

Peptide	Sequence	MRM transitions
T1	MD^{2}VTIQHPWFK	701.35>577.31 for the y4 ion, 467.90>577.31 for the y4 ion
T6	TVLD^{58}SGISEVR	588.32>490.26 for the y4 ion, 588.32>660.37 for the y6 ion, 588.32>747.40 for the y7 ion
T9	FVIFLD^{76}VK	490.79>360.21 for the y3 ion
T10	HFSPED^{84}LTVK	586.80>801.44 for the y7 ion,, 586.80>460.31 for the y4 ion, 586.80>347.22 for the y3 ion
T11	VQD^{91}D^{92}FVEHGK	429.55>341.19 for the y3 ion 429.55>415.23 for the y7 ion, 429.55> 454.28 for the y4 ion, 429.55>583.32 for the y5 ion, 429.55>682.39 for the y6 ion
T13	QD^{105}D^{106}HGYISR	364.17>595.32 for the y5 ion, 364.17>538.3 for the y4 ion, 364.17>375.24 for the y3 ion, 545.7>595.32 for the y5 ion
T18	IQTGLD^{151}ATHAER	437.89 > 400.20 for the y7 ion, 437.89 > 512.26 for the y4, and 437.89 > 375.2 for the y3 ion

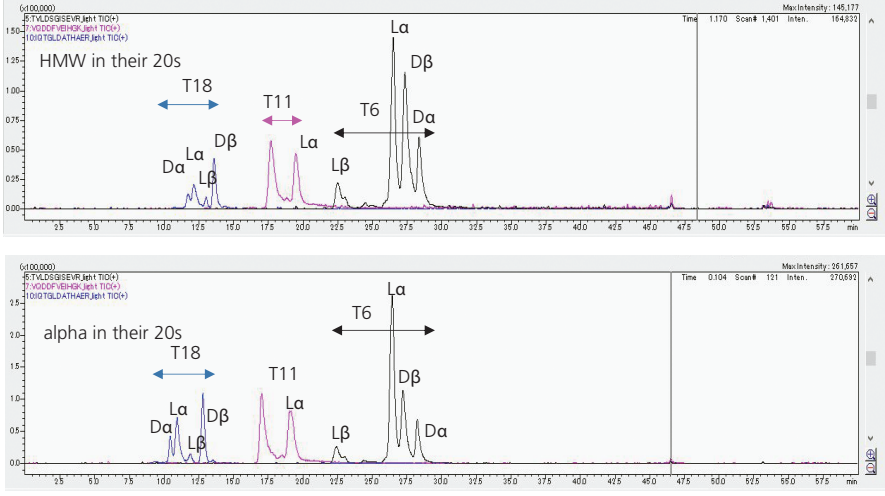

Fig. 6 Simultaneous analysis of Asp residues in αA-crystallin in lens using Micro-LC/LCMS-9030

MS system: LCMS-8060NX with Micro ESI-8060
LC system: Nexera Mikros (Trap & Elute system)
Column: Shim-pack MC PLONAS AQ-C18 2.7 µm (0.2 mm I.D. x 100 mmL) 90Å
Trap column: Shim-pack MCT LC18, 5 µm (0.3 mm I.D. x 5 mmL)
Mobile phase A：0.1 % Formic acid in water
Mobile phase B：0.1 % Formic acid in acetonitrile
Time program：5-15 % B in 60 min
Flow rate：5 µL / min
Column temp：40 °C

Table 2　LC and MRM conditions

Peptide	Sequence	MRM transitions
T6	TVLD^{58}SGISEVR	588.32>490.26 for the y4 ion, 588.32>660.37 for the y6 ion, 588.32>747.40 for the y7 ion
T11	VQD^{91}D^{92}FVEHGK	429.55>341.19 for the y3 ion 429.55>415.23 for the y7 ion, 429.55> 454.28 for the y4 ion, 429.55>583.32 for the y5 ion, 429.55>682.39 for the y6 ion
T18	IQTGLD^{151}ATHAER	437.89 > 400.20 for the y7 ion, 437.89 > 512.26 for the y4, and 437.89 > 375.2 for the y3 ion

Using this optimized transition information, simultaneous isomer analysis of the Asp residues in the soluble aggregate fraction (HMW) and in the αA-crystallin fraction within the normal α-crystallin fraction was performed on the eye lens of a person in their 20s. (Fig. 6). It was found that multiple separation of T6, T11, and T18 peptides in αA-crystallin separated Asp 58, 91/92, and 151 residues into 4 isomers. Each Asp isomer-containing peptide was identified according to the elution time of the synthetic peptide. The Asp isomer ratio of each residue can be easily calculated from the peak area ratio. As a result, when αA-crystallin in the normal α-crystallin fraction was transferred to the aggregated (HMW) fraction, the amount of normal La-Asp decreased together with the Asp 58 and Asp 151 residues, while the amount of isomer increased. In particular, the increase in Dβ-Asp was remarkable (Fig. 7). It proved that there was a close relation between agglomeration and increase in the isomerization rate for the Asp residue. As for the T11 peptide, it contained two Asp residues, hence it was difficult to examine the isomer ratio in detail.

3. Conclusions

Although it is now believed that D-amino acids in proteins increase with aging and are involved in age-related diseases, it is not easy to determine and quantify which amino acids in which proteins in aging tissues are D-isomerized. However, as demonstrated in this report, it has become possible to measure D-amino acids in proteins using LC-MS. We introduced a comprehensive analysis method in section 2-2 and a targeted method in section 2-3. With the first method, it is difficult to analyze low-expression proteins. This is because they are obscured by proteins with high expression levels. On the other hand, although target proteomics using the MRM method can be used to analyze these proteins, it cannot be applied to unknown samples because the MRM transitions need to be set in advance. In the analysis of the protein crystallin described in this report, determination of the multiple isomers of Asp and their relative ratios was comparatively easy. The detection sensitivity was increased about 10 times by using MicroLC. In the future, we believe that a combination of shotgun and target proteomics will be able to detect isomers of Asp in unknown proteins present in lesions such as in neurological diseases, arteriosclerosis, and aging tissues. The surprising fact that D-amino acids, while not synthesized by organisms, are produced in the course of aging over a single lifetime, has overturned a conventional biochemical concept. We expect that in the future the aging process may be expressed quantitatively from the new perspective of D-amino acids.

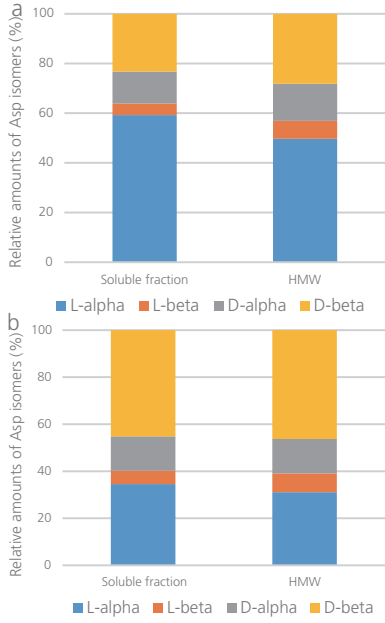

Fig. 7　Isomerization rate of Asp 58 (a) Asp 151 residue (b) in αA-crystallin present in soluble (WS) and aggregate (HMW) fractions of a lens from a 20-year-old human.

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First Edition: October 2020

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