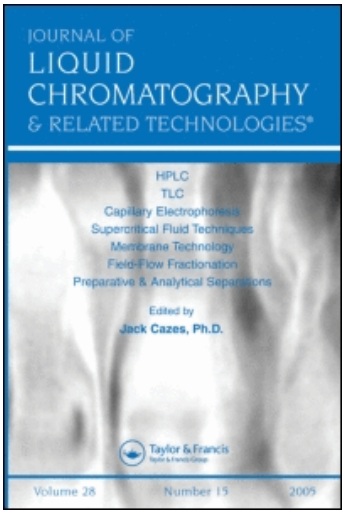

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A RAPID HPLC DETERMINATION OF THE ISOMERIZATION LEVEL OF ASP-RESIDUES WITHIN SYNTHETIC α-A-CRYSTALLIN FRAGMENTS

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ABSTRACT

We describe a rapid HPLC method for measuring the isomerization levels of Asp-residues within peptides. This method can detect the isomerization of Asp-residue at a level of less than 1% without performance of acid hydrolysis. Using this method, we further studied the isomerization of Asp-residues in the

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αA-crystallin f(55–65) fragment of sequence TVLDSGISEVR (T6). Four isomers of T6 peptides, which contain *L*-Asp, *L*-iso-Asp, *D*-Asp, and *D*-isoAsp, respectively, were synthesized. Following incubation of any one of these peptides, four possible isomers of T6 peptides were found in an aqueous buffer. The isoAsp/ Asp ratios of peptides in these solutions were found to reach 3.6–3.8 after incubation at 90°C for 72 hr.

INTRODUCTION

During the aging process of proteins and peptides, the carbonyl groups in *L*-Asp and *L*-Asn side chains cyclize to the nitrogen atom in the peptide bond of the adjacent amino acid residue, thereby resulting in stereoisomerization and structural isomerization. As shown in Fig. 1, an *L*-succinimide ring structure, which is a chemically unstable intermediate, is formed,[1,2] and then hydrolyzed into *L*-isoAsp residues, or back into *L*-Asp residues. The *L*-succinimidyl form is further converted into *D*-Asp or *D*-isoAsp residues, after isomerization to a *D*-succinimide cyclic intermediate.[3–7] These conversions are considered to be chemical reactions that naturally occur in the aging process of proteins. Recently, the isomerization of Asp-residues at specific sites was found to occur in *β*-amyloid proteins, which are related to Alzheimer's disease,[8] as well as in rat tubulin proteins.[9] Certain *L*-Asp residues in collagen, found in the spinal cord,

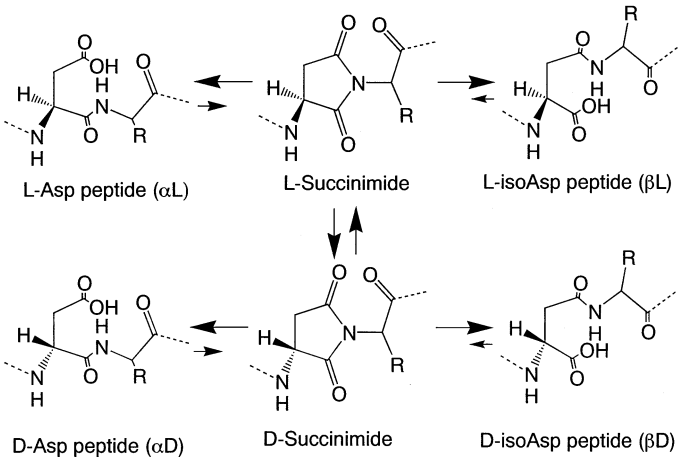

Figure 1. Pathways of spontaneous inversion, racemization, and isomerization of aspartyl residues via succinimide intermediates.

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are also isomerized at high ratios into *L*-isoAsp, *D*-Asp, or *D*-isoAsp residues. The estimation of collagen isomerization in urine has been found to be useful for the diagnosis of Paget's disease and osteoporosis.[10]

Determining the isomerization at certain Asp-residues in a protein for the purpose of the diagnosis requires a rapid analysis. However, the conventional method is time-consuming and not quantitative, because a protein is subjected to enzymolysis to fractionate the target peptide fragments, and then is subjected to either *D*/*L*-amino acid analysis for the estimation of *D*/*L* ratio of Asp-residues, or to sequencing analysis for the indirect identification of isomerized Asp-residues. Moreover, acid hydrolysis also causes additional racemization of amino acids, although only in a trace amount.[11]

In the present study, we aimed to develop a reversed-phase HPLC that enables simple and sensitive bulk analysis of peptides, in which certain Asp-residues are isomerized. We targeted the isomerization reactions of three kinds of peptides in αA-crystallin sequence, TVLDSGISEVR f(55–65), HFSPEDLTVK f(79–88), and IQTGLDATHAER f(146–157), because in vivo isomerization of these Asp-residues in αA-crystallin has been well defined by Fujii et al.[12,13]

EXPERIMENTAL

Peptide Synthesis

Three tryptic peptides of αA-crystallin, TVLDSGISEVR (T6), HFSPEDLTVK (T10), and IQTGLDATHAER (T18), were selected. For each of these three peptides, four types of isomeric peptides, which contain *L*-Asp, *L*-isoAsp, *D*-Asp, and *D*-isoAsp, respectively, were synthesized and designated as, for example, T6αL, T6*β*L, T6αD, and T6*β*D, respectively. Peptide synthesis was carried out with a Shimadzu PSSM-8 Peptide Synthesizer (Shimadzu, Kyoto, Japan). Fmoc–*L*-Asp(OtBu)–OH for the α*L*-peptides, Fmoc–*L*-Asp–OtBu for the *β*L-peptides, Fmoc–*D*-Asp(OtBu)–OH for the α*D*-peptides, Fmoc–*D*-Asp–OtBu for the *β*D-peptides, and other Fmoc-amino acid derivatives, were purchased from Watanabe Chemical Co., Ltd. (Hiroshima, Japan). The synthesized peptides were confirmed by subjecting them to amino acid composition analysis and amino acid sequencing analysis.

HPLC Separation of Peptide Isomers

The synthesized peptides were dissolved and mixed in water in amounts such that the final concentration would fall within the range of 0.210 μM to 25 μM, and subjected to separation and measurement by reversed-phase HPLC,

using EYELA PLC-5 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) as a pump and detector at a detection wavelength of 215 nm. The column used was a Develosil ODS-5 (4 mm i.d. × 150 mm, Nomura Chemical Co., Ltd, Aichi, Japan) and the flow rate was 1.0 mL/min. Acetonitrile–sodium phosphate solutions served as mobile phases. The mobile phases selected for analyzing T6, T10, and T18 peptide isomers were mixtures of acetonitrile and 15 mM sodium phosphate in the ratios of 15.5 : 84.5 (pH 5.1), 17.5 : 82.5 (pH 3.0), and 9.5 : 90.5 (pH 3.0), respectively. The injection volume was 20 μL.

Isomerization of Peptides

Each of the four types of Asp-residue isomeric peptides of T6, T10, and T18 (100 μM) was dissolved in a 50 mM phosphate buffer (pH 7.4). The resultant mixtures (200 μL each) were placed in glass tubes (7 × 80 mm), and the tubes were sealed by use of a gas burner. The mixtures were incubated at 37°C, 60°C, or 90°C, followed by freezing at −20°C, to thereby stop incubation and commence storage.

RESULTS

HPLC Separation of Four T6 Peptides

We studied the HPLC conditions for a separation of the four synthetic T6 peptides, T6αL, T6βL, T6αD, and T6βD, which have *L*-Asp, *L*-isoAsp, *D*-Asp, and *D*-isoAsp, respectively, at the Asp-position in their sequences. The effect of pH on separation was examined by use of acetonitrile–sodium phosphate eluate. The mobile phase had a sodium phosphate concentration of 15 mM, and its pH was varied within the range of 2.5–8.0. Figure 2 shows that the four T6 peptides were separated completely when pH fell within the range of 4.5–6.0 or was 8.5. Within the pH range of 4.5–6.0, almost identical chromatograms were obtained. Moreover, the change in pH was found to have no effect on the retention time of T6βL, whereas, it was found to greatly affect the retention time of T6αL. Overall, the retention time was found to become shorter with increasing pH, but tailing was observed at pH 8.5.

The HPLC conditions selected for analyzing four T6 peptides were as follows: the mobile phase was acetonitrile–15 mM sodium phosphate (pH 5.1) = 15.5 : 84.5, flow rate was 1.0 mL/min, and the column was a Develosil ODS-5 (4.0 × 150 mm). When four T6 peptides were subjected to this HPLC method, linearity ($r^2 = 0.995$) was demonstrated by their standard curves in the range 30–5000 pmol (T6αL), 12–5000 pmol (T6βL), 15–5000 pmol (T6αD) and 20–5000 pmol (T6βD). These detection limits were observed at 10, 4, 5, and 6 pmol, respectively. The accuracy was evaluated by five consecutive determinations at

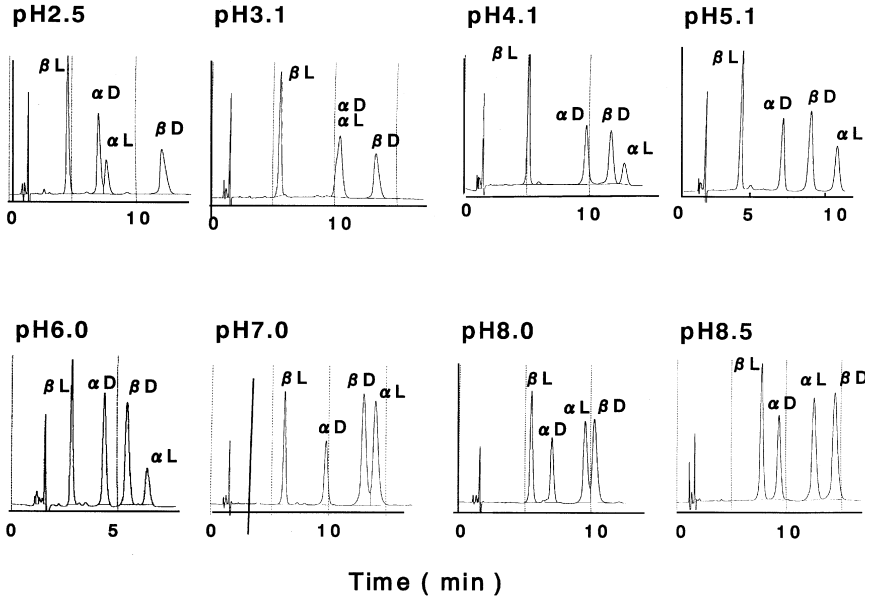


Figure 2. Effect of pH of the mobile phase on the separation of T6 peptide isomers. Sample: T6αL; Thr–Val–Leu–*L*-Asp–Ser–Gly–Ile–Ser–Glu–Val–Arg, T6*β*L; Thr–Val–Leu–*L*-iso-Asp–Ser–Gly–Ile–Ser–Glu–Val–Arg, T6αD; Thr–Val–Leu–*D*-Asp–Ser–Gly–Ile–Ser–Glu–Val–Arg, and T6*β*D; Thr–Val–Leu–*D*-isoAsp–Ser–Gly–Ile–Ser–Glu–Val–Arg. HPLC condition: pump, EYELA PLC-5; Detection, Abs. at 215 nm; column, Develosil ODS-5 (4 mm i.d. × 150 mm); flow rate, 1.0 mL/min; injection, 20 μL (1 nmol of each peptide); mobile phase, acetonitrile–15 mM sodium phosphate = 15.5 : 84.5 for lower pH eluents (pH 2.5–6.0), and 14.5 : 85.5 for higher pH eluents (pH 7.0–8.5).

200 pmol for each peptide. Good reproducibility of peak area (RSD < 5.0%) and retention time (RSD < 0.5%) for all four T6 peptides was shown. According to the HPLC analysis of the T6 peptides, Asp-isomeric peptides of each T10 or T18 were synthesized as the standard of HPLC analysis, and were analyzed using the selected mobile phase conditions that allowed simultaneous HPLC analysis (data not shown).

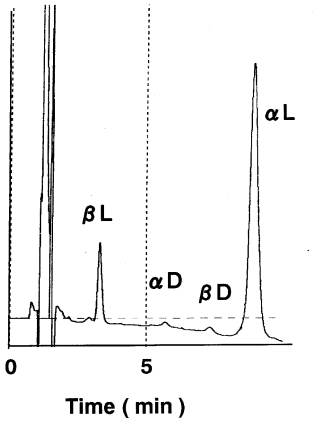
Measurement of Isomerization Levels of T6, T10, and T18 Peptides

To demonstrate the advantage of this HPLC analysis, T6αL that had been incubated at 37°C for 60 days, was analyzed using HPLC under the conditions

described above. Figure 3 shows the HPLC profiles of the treated T6αL peptide. New peaks appeared at the same retention time as in the three isomers of the T6 peptides; T6βL, T6αD, and T6βD. This method can detect T6αD at a level of less than 1% (<20 pmol).

We determined the isomerization levels of Asp-residues in T6αL, T10αL, and T18αL after incubation at 37°C for 45 days or 120 days (Fig. 4). Isomerization was observed in all three peptides, but these peptides differed greatly in *L*-isoAsp/*L*-Asp ratio. The levels of isomerization from *L*-Asp to *L*-isoAsp were found to be in the order of T6 > T18 > T10. We also confirmed that the Asp-isomerization from *L*-Asp to *L*-isoAsp occurred after incubation at 60°C or 90°C, in the same manner as that occurring after incubation at 37°C (data not shown).

We further studied the progress of isomerization of Asp-residue at 90°C in the four synthetic T6 peptides serving as starting materials (Fig. 5). When T6αL and T6αD served as the starting material, isomerization into T6βL or T6βD occurred at high ratios. In contrast, when T6βL or T6βD served as the starting material, isomerization into T6αL and T6αD occurred at lower levels. After incubation at 90°C for 72 hours, the four T6 peptides were found to have very similar isoAsp/Asp ratios; 3.8 when T6αL served as the starting material, 3.6 when T6βL or T6αD served as the starting material, and 3.7 when T6βD served as the starting material. This result shows, that the isoAsp/Asp ratio reached 3.6–3.8 when any of the T6 peptides served as the starting materials.



T6 peptide	% of isomers
αL	85.1 ± 0.6
αD	0.82 ± 0.02
βL	12.9 ± 0.2
βD	1.26 ± 0.03

Figure 3. Chromatogram and the isomerization data of T6α*L*-peptide. Synthetic T6α*L*-peptide (100 μM) was incubated with 50 mM phosphate buffer (pH 7.4) in a glass tube at 37°C for 60 days. Each number in the table indicates the mean ± S.D. ($n = 6$). Peptides and HPLC conditions are shown in Fig. 2.

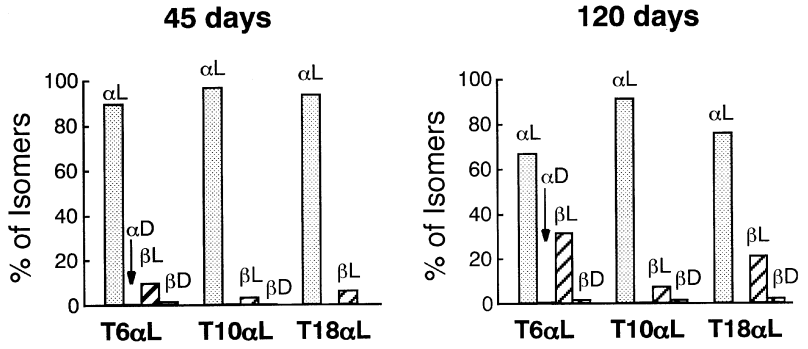


Figure 4. Isomerization of T6, T10, and T18α*L*-peptides after incubation at 37°C. Synthetic α*L*-peptides (100 μM) were incubated with 50 mM phosphate buffer (pH 7.4) in a glass tube at 37°C for 45 or 120 days. HPLC conditions are shown in Fig. 2.

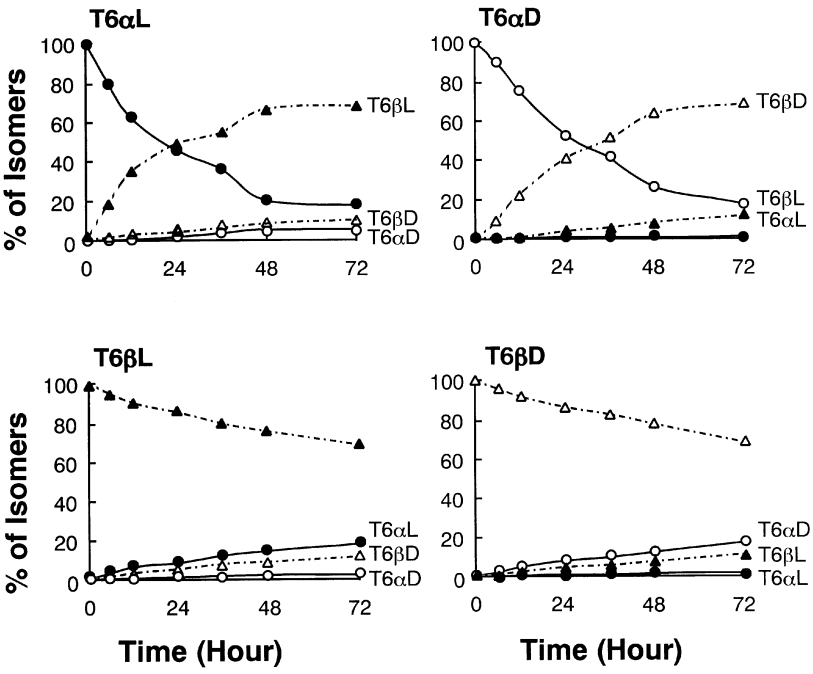


Figure 5. Isomerization of T6αL, T6*β*L, T6αD, and T6*β*D peptides after incubation at 90°C. Each of four synthetic T6 peptides (100 μM) was incubated with 50 mM phosphate buffer (pH 7.4) in a glass tube at 90°C for various times. HPLC conditions are shown in Fig. 2.

DISCUSSION

Conventional analysis for racemization of peptides requires acid hydrolysis followed by *D*/*L*-amino acid analysis, which causes additional racemization of amino acids, although in a trace amount.[14] Furthermore, the method is not able to analyze isoAsp residue, and amino acid sequencing analysis has to be carried out separately for this purpose. In amino acid sequencing analysis, isoAsp-containing peptides are confirmed indirectly by Edman degradation stopping at isoAsp-residues. In contrast to the conventional analyses, the present method constitutes simple and sensitive bulk analysis of Asp-residue isomerization using a reversed-phase HPLC. This method has a great advantage, in that it does not require acid hydrolysis, because peptides are analyzed directly; thus, it is free from racemization caused by acid hydrolysis and, as shown in Fig. 3, is capable of detection of the peptide containing *D*-Asp at a level of less than 1% (<20 pmol). Also, this method drastically reduces the time required for analysis and improves efficiency. By merely studying the HPLC conditions for separating the peptide isomers, this method can be potentially applied to any peptide, so long as its amino acid sequences are known, so as to enable synthesis of its standard. In addition, depending on the HPLC conditions, a plurality of isomerized peptides can be analyzed simultaneously, making this analysis a simple, precise, and useful method.

We demonstrated the usefulness of the present method by the determination of isomerization levels of Asp-residues in T6αL, T10αL, and T18αL. The levels of isomerization from *L*-Asp to *L*-isoAsp in these peptides were found to be in the order of T6αL > T18αL > T10αL, whereas those in human αA-crystallin were reported to be in the order of T18 > T6 > T10.[12] A possible explanation for the difference in the orders, is that T6 sequence within the protein is more restricted in motion and free T6 peptide fragment isomerizes more easily. The difference in the isomerization levels among the three peptides is ascribed to the difference of the amino acid residue in the neighboring carboxyl side-amino acid of Asp-residue. The neighboring amino acids in T6, T10, and T18 are Ser, Leu, and Ala, respectively. The carboxylic acid in the side chain of Asp-residues is considered, to encounter difficulty in forming a succinimide ring in the presence of a bulky side chain on the vicinal amino acids.[15–17] Our results were found to support this (Fig. 1) and suggest that isomerization of peptides is merely a chemical reaction.[5]

We found the isoAsp/Asp ratios reached 3.6–3.8 in all Asp-isomerized T6 peptides. Clarke et al reported that, in tetragastrin (WMDF–NH_2) peptides, a succinimide intermediate is converted into *L*-isoAsp or *L*-Asp residue at an isoAsp/Asp ratio of 4.[18] They also reported that VYPDGA peptide was isomerized into Asp-residue at an isoAsp/Asp ratio of 3.8.[2] These isoAsp/Asp ratios were found to be almost identical with our experimental results. Another

result obtained in the present study, is that Asp-residues in T6 peptides are isomerized to form all four isomers; T6αL, T6βL, T6αD, and T6βD, when any one of the four T6 peptides serves as the starting material. We also confirmed that all four T6 peptides were significantly isomerized, even at 37°C. Moreover, we have confirmed, that the same isomerization reaction occurs in synthetic T10 and T18 peptides (data not shown).

In the present study, we developed a method for quantifying isomerization of *L*-Asp residues into *L*-isoAsp, *D*-Asp, and *D*-isoAsp residues in the aging process of proteins. Isomerization of Asp- and Asn-residues in proteins is considered to be among the aggravating factors of Alzheimer's disease and cataracts. Moreover, the analysis of collagen isomerization in urine is expected to be useful for the diagnosis of Paget's disease and osteoporosis. The present method is capable of measuring the level of modification in proteins, such as isomerization or racemization in Asp-residues, through the analysis of Asp containing peptides derived from proteins. Since peptides are analyzed directly, this method is free from racemization caused by the conventional acid hydrolysis, and capable of pursuing the diagnosis, as well as functional changes in proteins.

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