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Journal of Pharmaceutical and Biomedical Analysis
30 (2003) 1825–1833

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Quantification of the isomerization of Asp residue in recombinant human αA-crystallin by reversed-phase HPLC

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Received 29 May 2002; received in revised form 8 August 2002; accepted 9 August 2002

Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

A method for determining the isomerization of Asp residues in proteins is described and demonstrated by quantifying the isomerization of Asp^{151} in recombinant human αA-crystallin. First, four types of dodecapeptide fragment (^{146}IQTGLD^{151}ATHAER^{157}) in which the Asp residue was either L-Asp, D-Asp, L-isoAsp or D-isoAsp were synthesized, and RP-HPLC conditions were established for their separation. Next, the Asp^{151}-containing peptide fragments isolated from the tryptic hydrolysate of recombinant αA-crystallin were analyzed under these conditions. New peaks, the retention times of which were the same as those of peptides containing D-Asp, L-isoAsp and D-isoAsp, were generated when αA-crystallin was incubated for 140 days at 37 °C. An amino acid composition, amino acid sequence, and enantiomeric analysis revealed that two peaks with retention times identical to those of peptides containing L-isoAsp and D-isoAsp represented dodecapeptide fragments containing L-isoAsp^{151} and D-isoAsp^{151}, respectively. RP-HPLC analysis under other condition suggested that the peak with retention time identical to that of peptide containing D-Asp represented dodecapeptide fragments containing D-Asp^{151}. The present method does not require acid hydrolysis, which generates further isomerization products as artifacts, and thus make possible the sensitive quantification of each type of Asp isomer individually at a specific site in a protein. In our analysis of the Asp^{151} residue in human αA-crystallin, the degree of isomerization from L-Asp to D-Asp can be determined to a level as low as 0.3%.
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Keywords: Enantiomerization; Isoaspartate formation; Eye lens; Crystallin; Recombinant protein

1. Introduction

Both structural isomerization (α- to β-linkage) and stereo-isomerization (L- to D-form) of Asp

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PII: S0731-7085(02)00525-3

residues has been found in various proteins and peptides, and these are though to occur by spontaneous chemical reactions under conditions of mild pH and temperature. The isomerized Asp residue is believed to arise through intramolecular rearrangement, such as via a succinimide intermediate [1]. The formation of succinimide is affected by both the primary and higher-order structures of protein [2–7]; succinimide is formed rapidly when a Gly, Ser, or Ala residue is a C-terminal side of the Asp residue, and when the polypeptide chain is highly flexible. Recently, the biological activities of proteins in which Asp residues are isomerized have been reported to be altered. Racemization of a certain Asp residue in Alzheimer amyloid β protein affects its aggregation properties, which is a key phenomenon in Alzheimer's disease [8,9], and isoAsp modification of the cytochrome c peptide fragment triggers autoimmune responses while the same peptide with L-Asp shows no response [10]. Moreover, protein L-isoaspartyl methyltransferase-deficient mice, in which isomerized Asp residues are accumulated at a level several times higher than in wild-type mice, undergo several tonic–clonic seizures, and die at a mean age of only 42 days [11–13]. These results show that the isomerization of Asp residues in proteins is a critical modification that is associated with various diseases.

Traditionally, the level of isomerization of Asp residues is determined through a combination of amino acid sequencing analysis, mass spectrometry, and enantiomeric analysis. This is both time-consuming and not quantitative, in that protein hydrolysis is required for enantiomeric analysis, which causes additional D-enantiomers [14,15], and isoAsp residues are confirmed indirectly through their resistance to Edman degradation. Since the methods that are used to quantify isomerized Asp residues in proteins play important roles in clinical diagnoses and in controlling the quality of pharmaceutical proteins, a rapid and quantitative method is required for the determination of isomerized Asp residues in proteins [16–21]. Cloos and Fledelius [16] have developed immunoassays to determine the level of isomerization for a certain Asp residue in collagen in the spinal cord, which has been found to be useful for the diagnosis of Paget's disease and osteoporosis. Aswad et al. [17] also pointed out the importance of monitoring the isoAsp level in pharmaceutical proteins, and developed a sensitive method for quantifying the isoAsp level with protein L-isoaspartyl methyltransferase without using radioisotopes [18]. Stevenson et al.[21] developed a method to quantify isoAsp residue in small peptides by fast atom bombardment mass spectrometry.

In the present study, we developed a reversed-phase HPLC technique that enabled the quantitative and sensitive analysis of the isomerized Asp residues in proteins. In this method, four types of Asp isomers (L-Asp, D-Asp, L-isoAsp and D-isoAsp) at a specific site in the protein can be quantified individually, and isomerization from L-form to D-form at a level as low as 0.3% can be detected since this method does not require acid hydrolysis. As a practical example, we analyzed the isomerization of the Asp^{151} residue in human αA-crystallin, which has been reported to show the highest level of isomerization among the Asp residues in this protein [22].

2. Materials and methods

2.1. Peptide synthesis

A dodecapeptide fragment, named T18 peptide (^{146}IQTGLD^{151}ATHAER^{157}; 18th tryptic fragment from the N-terminus) of human αA-crystallin was selected. Four types of T18 peptide in which the Asp residue was L-Asp, D-Asp, L-isoAsp or D-isoAsp were synthesized using a Shimadzu PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan). Fmoc-L-Asp(OtBu)-OH, Fmoc-D-Asp(OtBu)-OH, Fmoc-L-Asp-OtBu, and Fmoc-D-Asp-OtBu were purchased from Watanabe Chemical Co. Ltd. (Hiroshima, Japan) and used to synthesize the four types of Asp residue-isomerized peptide. The synthesized peptides were confirmed by analyses of the amino acid composition and the amino acid sequence, and by mass spectrometry.

2.2. HPLC separation

The synthesized peptides were dissolved and mixed in water at a final concentration of approximately 25 μM, and subjected to separation by reversed-phase HPLC using a Jasco HPLC system 880 (Nippon Bunko Co. Ltd, Tokyo, Japan) with a C18 column, Develosil ODS-UG-5 (4.6 mm i.d. × 150 mm, Nomura Chemical Co. Ltd, Aichi, Japan). For the separation of T18 peptides, the mobile phase was acetonitrile-sodium phosphate solutions with various pH. The standard condition for separating the four types of T18 peptides was acetonitrile-sodium phosphate solution (pH 3.0, 15 mM) (11:89, v/v) as a mobile phase with monitoring at 215 nm. The tryptic hydrolysate of αA-crystallin was separated with a linear gradient of 0–30% acetonitrile containing 0.1% TFA, over 30 min at a flow rate of 1 mL/min, with monitoring at 215 nm.

2.3. Expression of recombinant human αA-crystallin

A DNA fragment containing human αA-crystallin was obtained by PCR amplification from first-stranded cDNAs of human fetal brain (Clontech, CA) with a sense primer of 5′-CCATGGACGTGACCATCCAG-3′ and an antisense primer of 5′-GGCTGCTATCTAAAGGAGT-3′, with reference to Andley et al. [23]. The sense primer was designed to include the *Nco*I site and the underlining in the nucleotide sequence shows the restriction site. The PCR reaction was carried out for 40 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 60 s). The PCR product was gel-purified and subcloned by pBluescript TA vector (Strategene, CA). The *Nco*I–*Bam*HI digestion product of the DNA fragment of αA-crystallin was ligated into the *Nco*I–*Bam*HI site of the T7 expression vector pET-3d (Novagen, WI). The resulting construct was transformed into the *Escherichia coli* strain BL21 (DE3) pLysS (Novagen). Transformed *E. coli* cells were grown at 37 °C in *Lenox broth* medium containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol, and cultured at 37 °C until an A_{600} of 1.0 was reached. Isopropyl thio-β-D-galactoside was added at a final concentration of 0.3 mM and the specimen was further cultured for 5 h at 37 °C. *E. coli* cells were harvested by centrifugation at 5000 × *g* for 10 min, resuspended in 50 mM Tris/HCl (pH 7.8) containing 30 mM NaCl. The cell extract was obtained by freeze–thawing three times, and centrifuged at 20 000 × *g* for 10 min to prepare the supernatant.

2.4. Purification of recombinant human αA-crystallin

The supernatant of expressed *E. coli* cell suspension was separated by Sepharose CL-6B (20 mm i.d. × 420 mm) equilibrated with 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl. The same solution was used as the eluent at a flow rate of 0.6 ml/min. The fraction eluted in the first peak was recovered and applied to a DEAE TOYOPEARL column (20 mm i.d. × 100 mm; Tosoh Co. Ltd. Tokyo, Japan) equilibrated with 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl. αA-crystallin was eluted with a linear gradient of 10 mM phosphate buffer (pH 7.4) containing between 50 and 500 mM NaCl, and the fractions eluted with 150–300 mM NaCl were recovered. The sample was dialyzed against 50 mM phosphate buffer (pH 7.4), and the protein concentration was determined.

2.5. Incubation and tryptic digestion of recombinant human αA-crystallin

Recombinant αA-crystallin (2 mg/ml) dissolved in 50 mM phosphate buffer (pH 7.4) was placed into glass tubes (7 mm i.d. × 80 mm), and the tubes were sealed using a gas burner. The samples were heated at 90 °C for 15 min to inactivate proteolytic impurities, incubated at 37 °C for 140 days, and frozen at −30 °C to stop the reaction. These samples are designed 'aged' recombinant αA-crystallin. Recombinant αA-crystallin was digested with TPCK-treated trypsin (Sigma–Aldrich, MO) for 1 h at 37 °C at an enzyme-to-substrate ratio of 1:10 (mol/mol), and used as the sample to prepare T18 peptide.

2.6. Amino acid composition analysis

The peptides were hydrolyzed with gas-phase 6 N HCl in vacuo at 108 °C for 24 h. The hydrolysates were dried with a stream of N_2 gas, and dissolved with water. The amino acids were separated by HPLC with an ion-exchange column (MCI GEL AFR2PC, 6 mm i.d. × 50 mm, Mitsubishi Chemicals, Tokyo, Japan), detected as *o*-phthalaldehyde derivatives, and monitored with Jasco FP-920 (Nippon Bunko) with the excitation and the emission wavelengths of 344 and 443 nm, respectively.

2.7. Amino acid sequence analysis and L-isoaspartyl methyltransferase assay

The amino acid sequences were determined using Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin amino acid analyzer (Shimadzu PPSQ-21, Kyoto, Japan). ISOQUANT Isoaspartate Detection Kit (Promega, WI, USA) was used to determine the activity of L-isoaspartyl methyltransferase. HPLC analysis to detect *S*-adenosyl-L-homocystein was performed according to a method described by Schurter and Aswad [18].

2.8. Determination of the D/L ratio of Asp

The samples for the analysis of the amino acid composition were also used to determine the D/L ratio of Asp, and dissolved in 0.13 M borate buffer (pH 10.4). Samples were incubated with *o*-phthalaldehyde and *n*-*tert*-butyloxycarbonyl-L-cysteine to form diastereoisomers with fluorescence. The D/L ratio of Asp was determined using RP-HPLC with a Nova-Pak ODS column (3.9 mm i.d. × 300 mm, Waters, Tokyo, Japan), using fluorescence detection with the excitation and the emission wavelengths of 344 and 443 nm, respectively. The analysis was performed with acetonitrile–acetate buffer (pH 6.0, 0.1 M) (11:89, v/v) as a mobile phase, at a flow rate of 0.8 ml/min at 50 °C [24].

3. Results

3.1. Determination of the HPLC conditions for analysis of the isomerization of Asp residue in T18 peptide of human αA-crystallin

A dodecapeptide fragment, named T18 peptide (^{146}IQTGLD^{151}ATHAER^{157}; 18th tryptic fragment from the N-terminus) was chosen to analyze the isomerization of Asp^{151} residue in human αA-crystallin because this residue has been reported to show the highest level of isomerization among the Asp residues of αA-crystallin [22]. Four types of T18 peptide in which the Asp residue was L-Asp, D-Asp, L-isoAsp or D-isoAsp were synthesized, and these peptides were used as standards to establish the RP-HPLC condition to analyze the isomerization of Asp^{151}. The separation of these four types of T18 peptide was studied using mobile phase of various pH adjusted with phosphate solution. Studies in the pH range from 2.4 to 7.0 revealed that the four peptides could be separated from each other in phosphate solution at pH 2.7, 3.0 and 4.0 (Fig. 1). The elution order at pH 2.7 and 3.0 was peptides containing D-Asp, L-Asp, L-isoAsp, and D-isoAsp residues. At pH 3.3, the peptides containing L-Asp and L-isoAsp were retained equally. The elution order for peptides containing L-Asp and L-isoAsp was reversed at pH 4.0. When 0.01% TFA, with a pH of nearly 3, was used as the mobile phase instead of phosphate solution, the four peptides were not separated completely because of the tailing of peptide peaks (data not shown). Finally, the isomerization of Asp^{151} in T18 peptide was analyzed with acetonitrile–sodium phosphate solution (15 mM, pH 3.0) (11:89, v/v) as a mobile phase. Under this condition, quantification was shown to be linear ($r^2 > 0.9995$) from 50 to 50 000 pmol, and the limits of detection and quantification for these peptides were approximately 10–17 and 30–50 pmol, respectively.

3.2. Preparation of T18 peptides from recombinant human αA-crystallin

Recombinant human αA-crystallin was expressed in *E. coli* and purified by subsequent

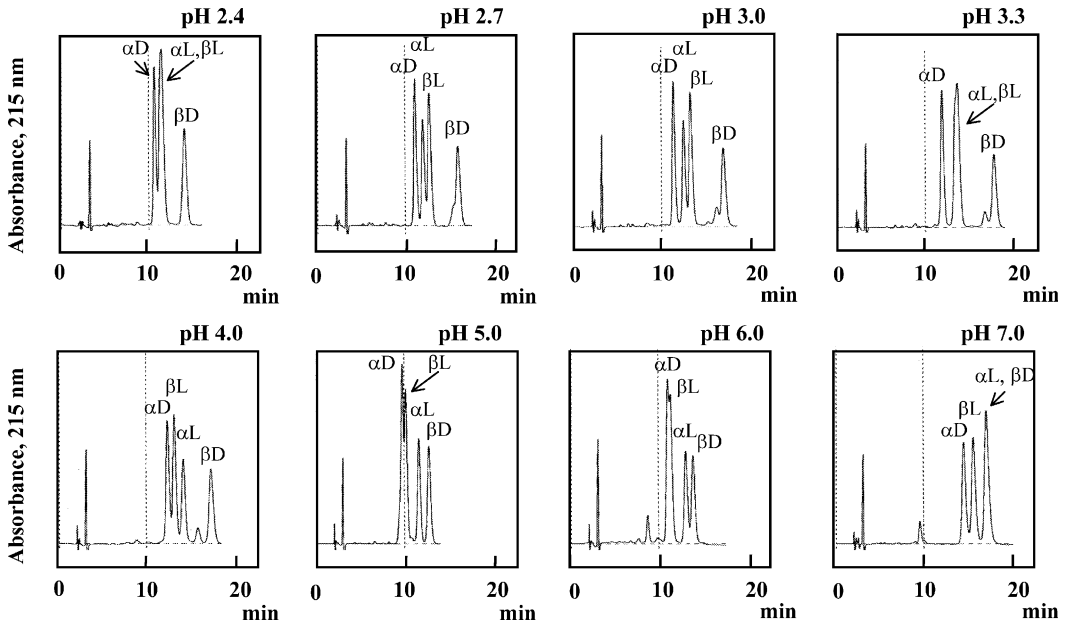

Fig. 1. Effect of pH on the separation of four T18 peptide isomers of human αA-crystallin. Four T18 peptide isomers in which the Asp residue was L-Asp, D-Asp, L-isoAsp or D-isoAsp were mixed, and separated by RP-HPLC with a mobile phase of various pH (pH 2.4–7.0). αL, βL, αD and βD indicate the peptides in which the Asp residue was L-Asp, L-isoAsp, D-Asp and D-isoAsp, respectively.

column operations (see Section 2), and the purity of recombinant αA-crystallin was found to be over 90%, judging from the densitometric analysis of SDS–PAGE gel stained with Coomassie brilliant blue (data not shown). DNA sequencing analysis revealed that the predict amino acid sequence generated by the insert DNA in expression vector was identical to that of human αA-crystallin (data not shown). It has reported that an initiation Met residue followed by an Asp residue could not be removed in *E. coli* [25], and we confirmed that the recombinant αA-crystallin still contained the initiation Met.

The purified recombinant αA-crystallin formed a large aggregate with a molecular mass of nearly 800 kDa (data not shown). Since this feature of recombinant αA-crystallin was very similar to that of native αA-crystallin [26], thus we used the recombinant αA-crystallin as a starting material to determine the isomerization of Asp^{151}. The purified αA-crystallin (2 mg/ml) was dissolved in 50 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 140 days. The aged αA-crystallin was digested by TPCK-treated trypsin, and the digestion product was separated by RP-HPLC with a linear gradient of 0–30% acetonitrile containing 0.1% TFA (Fig. 2). To confirm the retention of T18 peptides, a mixture of synthesized T18 peptides, in which the Asp residue was L-Asp, D-Asp, L-isoAsp or D-isoAsp, was separated under the same conditions. The fraction that included the four types of T18 peptide was recovered, concentrated, and used as a sample for analyzing the isomerization of Asp^{151}.

3.3. Assignment of the peaks corresponding to the four types of synthetic T18 peptide prepared from aged recombinant αA-crystallin

The samples isolated from aged recombinant αA-crystallin were separated by RP-HPLC under the conditions determined above (Fig. 3). Peaks with retention times identical to those of T18 peptides in which the Asp residue was L-Asp, D-Asp, L-isoAsp or D-isoAsp, designated peaks A, B, C and D (Fig. 3), were detected in the collected fractions of aged recombinant αA-crystallin incubated for 140 days at 37 °C. In the sample of

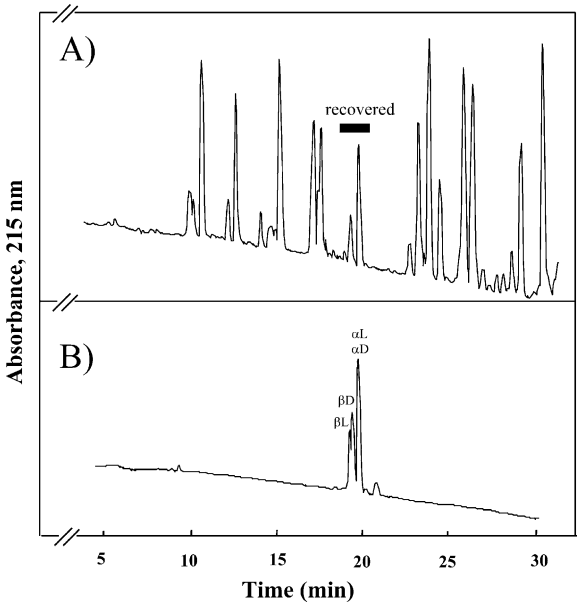

Fig. 2. Preparation of T18 peptides from tryptic hydrolysate of aged αA-crystallin. (A) Recombinant αA-crystallin incubated for 140 days at 37 °C was digested with TPCK-treated trypsin. The hydrolysate was separated by RP-HPLC with a linear gradient of 0–30% acetonitrile containing 0.1% TFA, over 30 min at a flow rate of 1 ml/min. The fraction indicated by the bar was recovered. (B) A mixture of the four T18 peptide isomers was separated by RP-HPLC using the same conditions as above.

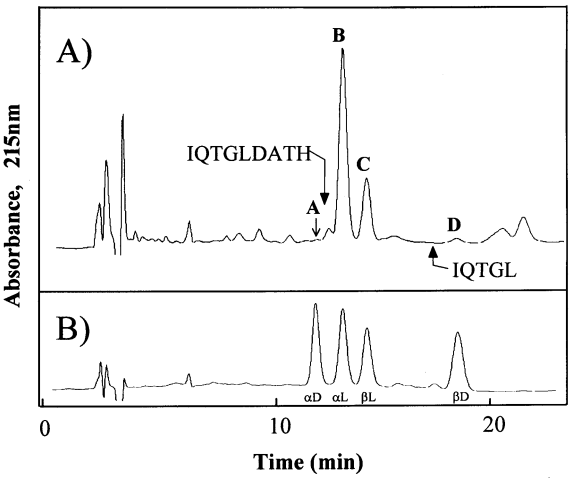

Fig. 3. Analysis of T18 peptides of aged αA-crystallin by RP-HPLC. (A) The fraction that included the four types of T18 peptide isolated from aged recombinant αA-crystallin was separated by RP-HPLC with acetonitrile–sodium phosphate solution (15 mM, pH 3.0) (11:89, v/v) as a mobile phase. The peaks with retention times that were the same as those of T18 peptides in which the Asp residue was D-Asp, L-Asp, L-isoAsp or D-isoAsp are marked A, B, C and D, respectively. The retention times of the peptides 'IQTGL' and 'IQTGLDATH' are also shown. (B) The mixture of four T18 peptide isomers was separated by RP-HPLC using the same conditions as above.

recombinant αA-crystallin without incubation, peaks A and D were not detected at all, and peak C was detected at less than 0.5% compared to peak B (data not shown). The four peaks were recovered, and their amino acid sequences were determined by automated Edman degradation (Fig. 4). Sequence analysis of peak B gave the sequence IQTGLDATHAER (Fig. 4A). Analysis of the amino acid composition of peak B supported the amino acid sequence (data not shown). Peaks C and D both had the same sequence as that of peak B, but with a large reduction in yield at cycle 6 (Fig. 4B and C). This result indicated that the Asp residues in these peaks had β-linkages. The amino acid composition of peak C corresponded to that for T18 peptide, and this peak fraction became a substrate for protein L-isoaspartyl methyltransferase (data not shown). Moreover, the retention times of peaks C and D differed from that of the peptide 'IQTGL' (Fig. 3). A D/L ratio analysis of Asp showed that peaks C and D consisted of L-Asp and D-Asp, respectively (data not shown). Thus, these results indicated that peaks C and D were the T18 peptide in which the Asp residue was L-isoAsp and D-isoAsp, respectively. Since there was too little peak A to determine its sequence and composition of amino acid, it was analyzed by RP-HPLC under other conditions (Fig. 5). While the elution order in the mobile phase at pH 4.1 differs from that under the standard conditions (pH 3.0), a peak with same area as peak A in the standard conditions appeared at the same retention time as the T18 peptide containing D-Asp. This strongly suggested that peak A was the T18 peptide in which the Asp residue was D-Asp. In summary, the four types of T18 peptide prepared from aged recombinant αA-crystallin could be well separated and quantified by RP-HPLC under the conditions determined using the standard peptides.

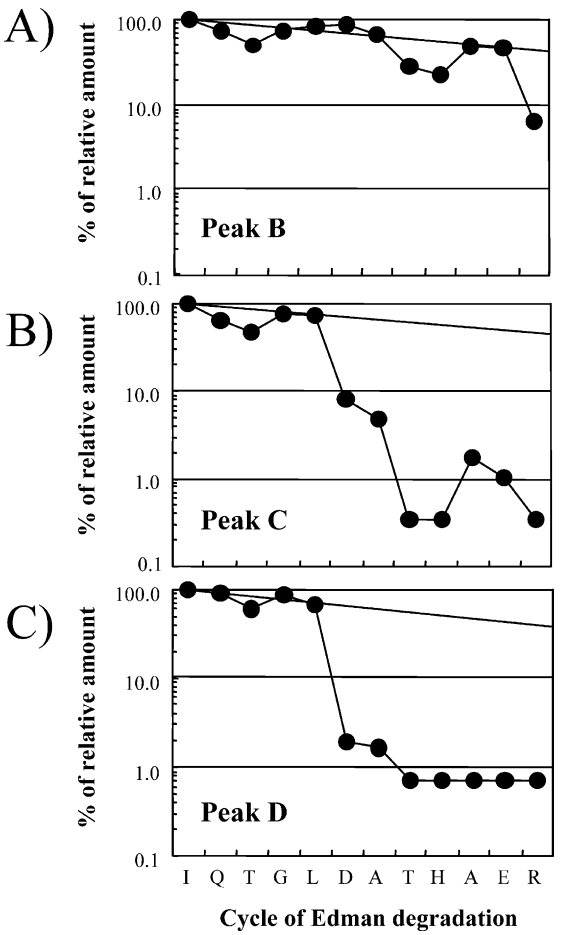

Fig. 4. Yields of peaks B, C and D isolated from aged αA-crystallin during amino acid sequence analysis (A) The sequence of peak B in Fig. 3 was analyzed by a pulsed-liquid protein sequencer. Yields are normalized to the first cycle (100%). (B) The relative yields of peak C in Fig. 3. (C) The relative yields of peak D in Fig. 3.

3.4. Quantification of the isomerization of Asp^{151} residue in recombinant human αA-crystallin

The peak areas corresponding to T18 peptides containing D-Asp, L-isoAsp or D-isoAsp were compared to that of T18 peptide containing L-Asp, and the ratios indicated both levels of isomerization and enantiomerization on condition that these peptides had an equal extinction coefficient (Fig. 6). The isoAsp/Asp and D-Asp/L-Asp ratio were 0.37 and 0.03, respectively, in the T18 peptide prepared from 140-day aged recombinant

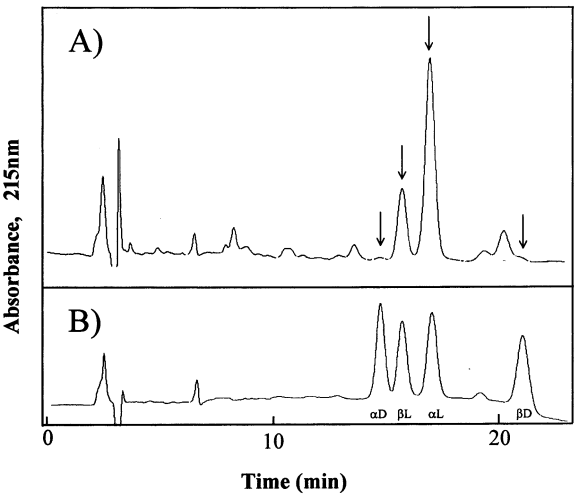

Fig. 5. Analysis of T18 peptides of aged αA-crystallin by RP-HPLC at a different pH. (A) The fraction that included the four T18 peptide isomers isolated from aged αA-crystallin was separated by RP-HPLC with acetonitrile-sodium phosphate solution (15 mM, pH 4.1) (11:89, v/v) as a mobile phase. The peaks with retention times that were the same as those of the four standard T18 peptide isomers are indicated by arrows. (B) The mixture of four T18 peptide isomers was separated by RP-HPLC using the same conditions as above.

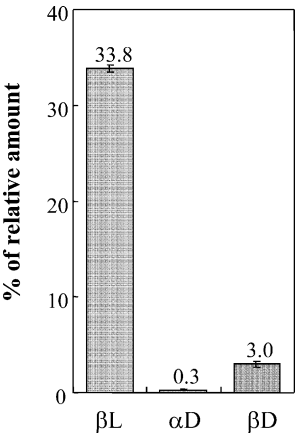

Fig. 6. Relative amounts of four types of T18 peptide in αA-crystallin incubated for 140 day at 37 °C. The amounts are normalized to area of the T18 peptide in which the Asp-residue is L-Asp (100%). The data are the average of three independent analyses, and the bars show the standard deviation.

αA-crystallin. Notably, this method showed that the relative amount of the peak corresponding to the T18 peptide containing D-Asp was 0.3%. Acid

hydrolysis, which is required in a conventional D/L-amino acid analysis, causes isomerization of Asp residues at an additive rate of 2 to 5% [14,15]. On the other hand, since the present method does not require acid hydrolysis, we can detect isomerization from the L-form to D-form at a level as low as 0.3%.

4. Discussion

The present method does not require acid hydrolysis and the isomerized Asp residue can be determined by RP-HPLC alone. Isomerization from the L-form to D-form can be detected at a level as low as 0.3% in this study. In the traditional method, such a level of isomerization can not be determined, since acid hydrolysis, which is required in a conventional D/L-amino acid analysis, causes isomerization of Asp residues at an additive rate of 2 to 5%. Recently, monitoring the isomerization of Asp residues has been reported to be important for clinical diagnosis and for controlling the quality of pharmaceutical proteins [16,17,19,20]. This method, which drastically reduces the time required for analysis and improves the sensitivity for detecting isomerized Asp residues, should assist in clinical decision-making.

However, the present method may not be suitable identifying which Asp or Asn residue in a protein is isomerized. The method can be used for a peptide only if its amino acid sequence is known, and the four peptide isomers are synthesized as a standard for HPLC analysis. The key to this method is whether the four peptide isomers can be separated by RP-HPLC. We have previously separated peptide isomers with various amino acid sequences: four types of other peptides in human αA-crystallin, ^{55}TVLDSGISEVR^{65} and ^{79}HFSPEDLTVK^{88}, and a peptide in human transferrin, ^{297}DSAHGFLK^{304} (underlined Asp residues vary between L-Asp, D-Asp, L-isoAsp and D-isoAsp), separated by RP-HPLC with acetonitrile-sodium phosphate solution as a mobile phase [27]. Cloos and Fledelius also show that the four C-terminal telopeptide isomers of type I collagen, ^{1209}AHDGGR^{1214} could be separated by RP-HPLC with acetonitrile–TFA as a mobile phase [16]. These results show that the high resolution RP-HPLC in the present method could make it possible to monitor various peptides in which an Asp residue is isomerized.

αA-crystallin is a major structural protein of the eye lens, and is associated with αB-crystallin. Since the proteins in the eye lens have very long half-lives, various posttranslational modifications, such as racemization, can occur [28–31]. Fujii et al. [22] showed that several Asp residues revert to the D-isomer during aging, and the Asp^{151} residue showed the highest level of isomerization in human αA-crystallin in vivo. Thus, the Asp^{151} residue in human αA-crystallin was used as a target to analyze isomerization of the Asp residue in the present study. We used recombinant αA-crystallin in this study, and showed that its feature was quite similar to that of native αA-crystallin. A recombinant protein expressed by *E. coli* was constructed by L-form amino acids, and we showed that no enantiomerization and very little isomerization occurred in the Asp^{151} residue in freshly prepared αA-crystallin. Thus, we and other groups have used recombinant proteins to examine in vitro posttranslational modification. Deamination and isoaspartate formation have been analyzed in two recombinant proteins: human growth hormone and human tissue plasminogen activator [4,32]. The use of recombinant proteins as starting materials should simplify the analysis of in vitro posttranslational modification.

5. Conclusion

The isomerization of Asp and Asn residues in proteins is reported to be a critical modification that is associated with various diseases, such as Alzheimer's disease and an autoimmune response, and mice that cannot repair an isomerized Asp residue reportedly have short life spans. Thus, the present method for quantification of isomerization by RP-HPLC should greatly facilitate the assessment of such protein modifications for clinical diagnosis and to control the quality of pharmaceutical proteins.

Acknowledgements

This work was supported in part by a grant-in-aid for the encouragement of young scientists (13771410) from The Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] P. Bornstein, G. Balian, Methods Enzymol. 47 (1977) 132–145.
[2] R. Tyler-Cross, V. Schirch, J. Biol. Chem. 266 (1991) 22549–22556.
[3] R.C. Stephenson, S. Clarke, J. Biol. Chem. 264 (1989) 6164–6170.
[4] M.V. Paranandi, A.W. Guzzetta, W.S. Hancock, D.W. Aswad, J. Biol. Chem. 269 (1994) 243–253.
[5] N. Fujii, K. Harada, Y. Momose, N. Ishii, M. Akaboshi, Biochem. Biophys. Res. Commun. 263 (1999) 322–326.
[6] K. Petel, R.T. Borchardt, Pharm. Res. 7 (1990) 787–793.
[7] M. Xie, J. Aube, R.T. Borchardt, M. Morton, E.M. Topp, D. Vander Velde, R.L. Schowen, J. Peptide Res. 56 (2000) 165–171.
[8] A.E. Roher, J.D. Lowenson, S. Clarke, C. Wolkow, R. Wang, R.J. Cotter, I.M. Reardon, H.A. Zurcher-Neely, R.L. Heinrikson, M.J. Ball, B.D. Greenberg, J. Biol. Chem. 268 (1993) 3072–3083.
[9] T. Tomiyama, S. Asano, Y. Furiya, T. Shirasawa, N. Endo, H. Mori, J. Biol. Chem. 269 (1994) 10205–10208.
[10] M.J. Mamula, R.J. Gee, J.I. Elliott, A. Sette, S. Southwood, P.-J. Jones, P.R. Blier, J. Biol. Chem. 274 (1999) 22321–22327.
[11] E. Kim, J.D. Lowenson, D.C. MacLaren, S. Clarke, S.G. Young, Proc. Natl. Acad. Sci. USA 94 (1997) 6132–6137.
[12] A. Yamamoto, H. Takagi, D. Kitamura, H. Tatsuoka, H. Nakano, H. Kawano, H. Kuroyanagi, Y. Yahagi, S. Kobayashi, K. Koizumi, T. Sakai, K. Saito, T. Chiba, K. Kawamura, K. Suzuki, T. Watanabe, H. Mori, T. Shirasawa, J. Neurosci. 18 (1998) 2063–2074.
[13] J.D. Lowenson, E. Kim, S.G. Young, S. Clarke, J. Biol. Chem. 276 (2001) 20695–20702.
[14] R. Liardon, S. Ledermann, U. Ott, J. Chromatogr. 203 (1981) 385–395.
[15] H. Frank, W. Woiwode, G. Nicholson, E. Bayer, Liebigs Ann. Chem. 1981 (1981) 354–365.
[16] P.A. Cloos, C. Fledelius, Biochem. J. 345 (2000) 473–480.
[17] D.W. Aswad, M.V. Paranandi, B.T. Schuter, J. Pharm. Biomed. Anal. 21 (2000) 1129–1136.
[18] B.T. Schurter, D.W. Aswad, Anal. Biochem. 282 (2000) 227–231.
[19] C.L. Stevenson, R.J. Anderegg, R.T. Borchardt, J. Pharm. Biomed. Anal. 11 (1993) 367–373.
[20] N.P. Bhatt, K. Patel, R.T. Borchardt, Pharm. Res. 7 (1990) 593–599.
[21] C.L. Stevenson, T.D. Williams, R.J. Anderegg, R.T. Borchardt, J. Pharm. Biomed. Anal. 10 (1992) 567–575.
[22] N. Fujii, K. Satoh, K. Harada, Y. Ishibashi, J. Biochem. 116 (1994) 663–669.
[23] U.P. Andley, S. Mathur, T.A. Griest, J.M. Petrash, J. Biol. Chem. 271 (1996) 31973–31980.
[24] A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi, T. Hayashi, J. Chromatogr. 582 (1992) 41–48.
[25] H. Dalboge, S. Bayne, J. Pedersen, FEBS Lett. 266 (1990) 1–3.
[26] G.J. Wistow, J. Piatigorsky, Ann. Rev. Biochem. 57 (1988) 479–504.
[27] M. Kaneda, K. Nakagomi, Y. Sadakane, T. Yamazaki, T. Tanimura, T. Akizawa, N. Fujii, Y. Hatanaka, J. Liq. Chromatogr. Rel. Technol. 25 (2002) 2445–2454.
[28] P.M. Masters, J.L. Bada, J.S. Zigler, Jr, Nature 268 (1977) 71–73.
[29] P.M. Masters, J.L. Bada, J.S. Zigler, Jr, Proc. Natl. Acad. Sci. USA 75 (1978) 1204–1208.
[30] P.J.T.A. Groenen, P.R.L.A. van den Ijssel, C.E.M. Voorter, H. Bloemendal, W.W. de Jong, FEBS Lett. 269 (1990) 109–112.
[31] N. Fujii, Y. Ishibashi, K. Satoh, M. Fujino, K. Harada, Biochim. Biophys. Acta 1204 (1994) 157–163.
[32] B.A. Johnson, J.M. Shirokawa, W.S. Hancock, M.W. Spellman, L.J. Basa, D.W. Aswad, J. Biol. Chem. 264 (1989) 14262–14271.