A Rapid, Comprehensive Liquid Chromatography-Mass Spectrometry (LC-MS)-based Survey of the Asp Isomers in Crystallins from Human Cataract Lenses*§

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Background: Asp isomers in lens crystallins are one of the triggers of cataracts.

Results: Multiple highly isomeric Asp sites in insoluble crystallins from cataract lenses were identified by LC-MS using the corresponding synthetic peptides as standards.

Conclusion: Asp isomers induce insolubilization of crystallin, leading to cataracts.

Significance: This study opens up a new field of protein biochemistry in age-related diseases.

Cataracts are caused by clouding of the eye lens and may lead to partial or total loss of vision. The mechanism of cataract development, however, is not well understood. It is thought that abnormal aggregates of lens proteins form with age, causing loss of lens clarity and development of the cataract. Lens proteins are composed of soluble α-, β-, and γ-crystallins, and as long lived proteins, they undergo post-translational modifications including isomerization, deamidation, and oxidation, which induce insolubilization, aggregation, and loss of function that may lead to cataracts. Therefore, analysis of post-translational modifications of individual amino acid residues in proteins is important. However, detection of the optical isomers of amino acids formed in these proteins is difficult because optical resolution is only achieved using complex methodology. In this study, we describe a new method for the analysis of isomerization of individual Asp residues in proteins using LC-MS and the corresponding synthetic peptides containing the Asp isomers. This makes it possible to analyze isomers of Asp residues in proteins precisely and quickly. We demonstrate that Asp-58, -76, -84, and -151 of αA-crystallin and Asp-62 and -96 of αB-crystallin are highly converted to Lβ-, Dβ-, and ω-isomers. The amount of isomerization of Asp is greater in the insoluble fraction at all Asp sites in lens proteins, therefore indicating that isomerization of these Asp residues affects the higher order structure of the proteins and contributes to the increase in aggregation, insolubilization, and disruption of function of proteins in the lens, leading to the cataract.

A cataract is caused by clouding of the eye lens and may lead to blindness. By age 80, more than 90% of people either have a cataract or have had cataract surgery. Although cataracts are among the most common age-related diseases, the mechanism of cataract development is not well understood. However, it is thought that proteins of the eye lens aggregate abnormally, resulting in clumping that scatters the light and interferes with focusing on the retina. Human lens proteins are mainly composed of α-, β-, and γ-crystallins, and the overall structure, stability, and short range interactions of these proteins are thought to contribute to the transparent properties of the lens. α-Crystallin is a large molecule with a molecular mass of ~800 kDa and comprises two kinds of polypeptides: αA and αB. Because each αA- or αB-crystallin monomer has a mass of ~20 kDa, the α-crystallin molecule is an aggregate containing ~40–50 subunits. The β/γ-crystallin superfamily comprises oligomeric β-crystallin and monomeric γ-crystallin (1). αA- or αB-crystallin are members of the small heat-shock protein family and function as molecular chaperones to protect β- and γ-crystallins from aggregation (2). Along with β- and γ-crystallin, lens crystallin accounts for about 90% of total water-soluble (WS) protein in a highly concentrated form and constitutes the refractive medium. Lenses are constantly subjected to UV light and oxidative stress, and therefore damaged proteins accumulate in water-insoluble (WI) fractions in the lens because there is no turnover of the lens proteins. It is well known that WI proteins increase in aged and cataractous lenses (3). Furthermore, the lens crystallins undergo various post-translational modifications such as isomerization and inversion of aspartic acid (Asp) residues, that is Lβ-, Dβ-, and ω-formation (4–6); deamidation of asparagine (Asn) or glutamine (Gln) residues (7–11); disulfide bond of cysteine (12); oxidation of methionine or tryptophan (13, 14); backbone cleavage (15); phosphorylation (16); and glycation (17) during the aging process. These modifications may induce a decrease in crystallin solubility, alter lens transparency, diminish vision, and lead to development of a cataract. Indeed, there is a strong relationship among post-translational modifications, aggregation, and loss

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§ The abbreviations used are: WS, water-soluble; RP, reverse-phase; WI, water-insoluble; ω, ratios of isomerization in the peptides; Fmoc, N-(9-fluorenylmethoxycarbonyl); Asp(OtBu)-OH, aspartic acid β-t-buty1 ester.
of solubility of crystallin. Of the post-translational modifications, we have proposed that the appearance of \( \delta \beta \)-Asp residues may be responsible for the change in the higher order structure and the loss of function of crystallins. This is due to the fact that if \( \delta \)-Asp is formed in the protein the configuration of the Asp residue would be opposite to the usual configuration, and hence this would induce a change in the higher order structure of the protein. In addition, the \( \beta \)-linkage produced with Asp formation may affect the quaternary structure of crystallin because the main chain of the protein would be elongated. Therefore, the presence of the Asp isomers may be one of the triggers of the insolubilization, abnormal aggregation, and induction of partial unfolding of protein and lead to a disease state. In fact, in our previous study, we clearly showed that \( \alpha \)-crystallin containing large amounts of \( \delta \beta \)-Asp undergoes abnormal aggregation to form massive and heterogeneous aggregates, leading to loss of its chaperone activity (18).

Deamidation of Asn or Gln introduces a negative charge at physiological pH by replacing an amide with a carboxyl group to form Asp or Glu, respectively, and causes some isomerization (4, 19). Isomerization and deamidation are major non-enzymatic post-translational modifications of proteins under physiological conditions. We have clarified the mechanism by which \( \delta \)-Asp residues are spontaneously formed in proteins (4–6). As shown in Fig. 1, the simultaneous formation of \( \beta \)- and \( \delta \)-Asp residues in the protein could be explained as follows. (i) When the carbonyl group of the side chain of the \( \lambda \alpha \)-Asp/\( \lambda \)-Asn residue is attacked by the nitrogen of the amino acid residue following the Asp residue, \( \lambda \)-succinimide is formed by intramolecular cyclization. (ii) \( \lambda \)-succinimide may be converted to \( \delta \)-succinimide through an intermediate that has the prochiral \( \alpha \)-carbon in the plane of the ring. (iii) Protonation of the intermediate may proceed from the upper or lower side of the plane in an ordinary peptide or protein. (iv) \( \delta \)- and \( \lambda \)-succinimides are hydrolyzed at either side of their two carbonyl groups, yielding both \( \beta \)- and \( \alpha \)-Asp residues, respectively. Thus, four isomers, \( \lambda \alpha \)-Asp, \( \lambda \beta \)-Asp, \( \delta \alpha \)-Asp, and \( \delta \beta \)-Asp, are simultaneously formed in the protein. The rate of succinimide formation is expected to depend on the neighboring residue of the Asp. When the neighboring amino acid of the Asp residue has a small side chain such as glycine, alanine, or serine, the formation of succinimide occurs easily because there is no steric hindrance. The deamidation of Asn residues in proteins is detected easily because the formation of Asp from Asn induces a +1 mass shift. Therefore, this mass shift caused by deamidation can be easily detected using mass spectrometry analysis. However, it was not possible to detect the \( \delta \)- or \( \beta \)-Asp isomers in proteins by mass spectrometry because the mass of the peptide containing Asp isomers is exactly the same as the normal peptide. Recently, however, we found that MS/MS analysis using postsource decay with a curved field reflectron could distinguish between the \( \beta \)-Asp- and \( \alpha \)-Asp-containing peptides (20). The relative content of \( \beta \)-Asp in a peptide was successfully estimated from the unique ratio, \( y_\beta /y_\alpha + 1 \), derived from tryptic peptides of a protein. However, there remained the problem of distinguishing between \( \delta \)-Asp and \( \lambda \)-Asp in the peptide precisely and quickly. In conventional analysis of Asp isomers, the following steps are required to determine the site of \( \delta \beta \)-Asp in a protein. (i) The protein is digested with a protease such as trypsin. (ii) The resulting peptides are separated by reverse-phase high performance liquid chromatography (RP-HPLC). (iii) The peptides are identified by mass analysis and/or protein sequencing. (iv) The \( \alpha \)- or \( \beta \)-isomer of the identified peptides is determined by Edman degradation reaction. (v) The \( \delta / \lambda \) ratios of the Asp residues are determined after hydrolysis of peptides with \( 6 \) N HCl and derivatization. (vi) The diastereoisomers are analyzed by RP-HPLC, and the \( \delta / \lambda \) ratio of amino acids is determined by analysis of the respective peak areas. Hence, the analysis of the isomerization of Asp residues in a protein is a technically demanding process.

In the present study, we propose a new method of analysis for determining the peptides containing Asp isomers at individual sites and for detecting inverted Asp residues in any protein by using LC-mass spectrometry (MS) systems. The advantages of the method are as follows. 1) There is no need for purification of lens proteins from WI and WS fractions. 2) There is no need for the complicated analytical steps iii–vi as described above. Here we present a method for quickly and easily distinguishing \( \delta \)- and \( \lambda \)-Asp-containing peptides in non-purified proteins using
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LC-MS. This is a groundbreaking method for the detection of Asp isomers in proteins.

EXPERIMENTAL PROCEDURES

Preparation of WI and WS Proteins from Human Lenses—Lens samples (one lens sample each) from elderly individuals (60–80 years old) were homogenized in 20 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA by ultrasonication and fractionated into WI and WS fractions by centrifugation at 16,000 × g for 20 min at 4°C. The WI proteins were dissolved in 8 mM urea, 50 mM Tris/HCl, pH 7.8, 1 mM CaCl2, and then the urea concentration was diluted to less than 1 M in 50 mM Tris/HCl, pH 7.8, 1 mM CaCl2 buffer before enzymatic digestion.

Enzymatic Digestion of WI and WS Proteins—The WI proteins were digested with trypsin (sequencing grade modified trypsin, Promega) for 17 h at 37°C in 50 mM Tris/HCl, pH 7.8, 1 mM CaCl2 buffer and then the urea concentration was diluted to less than 1 M in 50 mM Tris/HCl, pH 7.8, 1 mM CaCl2 buffer before enzymatic digestion.

Isolation of Tryptic Peptides of the WI and WS Proteins from Human Lenses by LC-MS—LC used a nanoflow HPLC system (Paradigm MS54, Michrom Bioresources). MS was performed on an ion trap system (LCQ Fleet, Thermo). The peptides (about 500 ng) resulting from digestion with trypsin were separated by nanoflow RP-HPLC using a C18 column (L-column, 250 mm × 1.0 mm, Chemicals Evaluation and Research Institute, Japan) with a linear gradient of 5–60% acetonitrile in the presence of 0.1% formic acid at a flow rate of 0.5 μl/min and analyzed by Proteome Discoverer 1.0 software. MS analysis was carried out alternating between full MS and MS/MS scans. The MS/MS scan used the collision-induced dissociation mode with dynamic exclusion function.

Synthesis of Peptides Containing Four Different Asp Isomers—Synthetic peptides containing four different Asp isomers were made using Fmoc solid-phase chemistry using an automated solid-phase peptide synthesizer (21) (PSSM-8, Shimadzu, Japan). Fmoc-1-aspartic acid β-t-butyl ester (Fmoc-1-Asp(OtBu)-OH), Fmoc-β-Asp(OtBu)-OH, Fmoc-γ-Asp-OtBu, and Fmoc-δ-Asp-OtBu were used as building blocks to synthesize L−, D−, β−, and δ−isomers, respectively. The coupling reaction was carried out using each Fmoc amino acid (10 eq), 1H-benzo-triazol-1-yl-oxy-tri(pyridylidino) phosphonium hexafluorophosphate (10 eq), 1-hydroxybenzotriazole (10 eq), and N-methylmorpholine (7.5 eq) in dimethylformamide (22). The N-terminal Fmoc group was deblocked with 20% piperidine in dimethylformamide. Simultaneous cleavage of the peptide from the resin and removal of the protective groups were achieved by treatment with a mixture containing 90% trifluoroacetic acid (TFA), 5% 1,2-ethanediol, and 5% thioanisole for 2 h. The cleavage of the peptide containing Arg and its protective groups from the resin was performed with 82.5% TFA, 5% water, 5% thioanisole, 3% ethylmethysulfide, 2.5% 1,2-ethanediol, and 2% thiophenol for 6 h. The cleavage of the peptide containing Trp and its protective groups from the resin was carried out with the above reagents plus 1 mg/ml 2-methylindole. The crude peptides were purified by RP-HPLC using a C18 column (Capcell Pak C18, 30 × 250 mm; Shiseido, Japan) with a linear gradient of 0–50% acetonitrile in the presence of 0.1% TFA at a flow rate of 3.0 ml/min with monitoring at 230 or 280 nm. The purity of each peptide was confirmed to be >95% by analytical RP-HPLC and mass spectrometry. The yields of the purified peptides were about 50%.

RESULTS

A New General Survey of Isomeric Asp Residues in Crystallins by LC-MS—Fig. 2 shows a set of typical LC-MS chromatograms from tryptic digests of the WI and WS proteins derived from the lens of a 64-year-old individual. Fig. 2, a-1 and a-2, show typical full LC-MS chromatograms of the tryptic peptides from the WI and WS proteins, respectively, derived from the lens of a 64-year-old donor. The mass numbers of these peaks were measured and then analyzed by MS/MS, and then all peaks were identified using the Proteome Discover 1.0 software attached to this LC-MS system. Generally, each peptide would be expected to elute as one peak with one mass number. However, some peptides that contain Asp residues were often separated into multiple peaks, and they eluted at different retention times during the LC-MS run even though it was entirely the same peptide. For example, the peptide predicted to be positions 55–65 of αA-crystallin (αA 55–65; TVLDGISEVR; [M + 2H]2+ = 588.3) as identified by the database was mainly separated into four different peaks and eluted at different elution times between 32 and 40 min of the LC-MS run as shown in Fig. 2, b-1 and b-2. Fig. 2, b-1 and b-2, show the peaks from WI and WS proteins that have an MS range of 588–589.5 m/z extracted from the full mass data shown in Fig. 2, a-1 and a-2. As shown in Fig. 2b, the number of peptides with [M + 2H]2+ = 588.3 (Fig. 2, b-1) was much greater from WI protein than that from WS protein (Fig. 2, b-2). Similarly, we extracted multiple peptides with an MS range of 745.5–750 from the full MS data in Fig. 2a, and these data are shown in Fig. 2c. These peaks correspond to residues 57–69 of αB-crystallin (αB 57–69; APSWFDTGLEMR; [M + 2H]2+ = 748.8) peptide. The peptide with [M + 2H]2+ = 748.8 from WI protein (Fig. 2, c-1) was mainly separated into four peaks, whereas the peptide [M + 2H]2+ = 748.8 from WS protein (Fig. 2, c-2) was mainly separated into two peaks. We further analyzed these multiple peptides from the WI fraction of the 64-year-old donor using MS/MS analysis. Fig. 3a-A shows the LC-MS chromatogram of αA 55–65 (TVLDGISEVR; [M + 2H]2+ = 588.3) from the WI fraction of lens proteins from the 64-year-old donor. Fig. 3, a-1 to a-7, correspond to the MS/MS analysis of peak numbers 1–7 in Fig. 3a-A, respectively. Fig. 3, a-1 to a-7, clearly show that these MS/MS analyses are entirely the same. Therefore, peak numbers 1–7 in Fig. 3a-A were all assigned as the peptide TVLDGISEVR, contained in four main peaks (peak numbers 1, 4, 5, and 7) and minor peptides (peak numbers 2, 3, and 6). Fig. 3b-B shows the LC-MS chromatogram of αB 57–69 (APSWFDTGLEMR; [M + 2H]2+ = 748.8) from the WI fraction of lens proteins from the 64-year-old donor. Fig. 3, b-1 to b-7, correspond to the MS/MS analysis of peak numbers 1–7 in Fig. 3b-B, respectively. As shown in Fig. 3, b-1 to b-7, b-1 to b-5 are entirely the same, but b-6 and b-7 are different from b-1 to b-5.
The results clearly show that peak numbers 1–5 in Fig. 3 are all αB 57–69 but that peaks 6 and 7 are not the αB 57–69 peptide but rather are unknown peptides.

Similar multiple peak separation of the various peptides containing Asp residues was found for both WI and WS proteins as shown in Table 1. This separation was thought to be due to the presence of different Asp, Glu, or Pro isomers and deamidation of Asn/Gln in the peptides. MS analysis should be able to distinguish deamidation of Asn/Gln on the basis of a mass difference of 1, but the peaks obtained from the LC-MS were detected as divalent or trivalent ions, and therefore a mass difference of 1 would be detected as only 0.5 or 0.3. This small difference could not accurately be detected in our LC-MS system. Therefore, we analyzed the peptides containing Asn/Gln by MS/MS to distinguish between deamidated peptides and isomerized peptides.

The above equation indicates that the closer the rI value is to 1.0 the less isomerization occurs in the peptide. The rI values are indicators of the presence of the isomeric peptides, and the smaller the rI, the more isomers are present. Table 1 shows the rI values of the tryptic peptides from WI-αA-crystallin and WI-βB-crystallin. The results clearly indicate that isomerization occurs in the proteins of the WI fraction in the lenses of donors of any age. In the WI fraction, the peptides 146–157, 55–65, 89–99, 104–112, and 22–49 of WI-αA-crystallin and 57–69 and 108–116 of WI-βB-crystallin contain the isomers with highest percentages. The amount of Asp isomers in β- and γ-crystallin is smaller than in αA- or αB-crystallins.

Identification of Asp Isomers in Crystallins from WI and WS Fractions of Lens—The peptide 55-7VLDGISEVR65 was separated into four main peaks and three minor peaks from both WI and WS proteins (Fig. 2b). This separation was predicted to be due to the presence of Asp residues in the peptide. The rI values of the peptides from the WI faction are smaller than those of the peptides from the WS fraction in all samples. The isomeric Asp residues are much more frequent in the WI fraction than in the WS fraction in all crystallins. The results clearly indicate that isomerization occurs in the proteins of the WI fraction in the lenses of donors of any age. In the WI fraction, the peptides 146–157, 55–65, 89–99,104–112, and 22–49 of WI-αA-crystallin and 57–69 and 108–116 of WI-βB-crystallin contain the isomers with highest percentages. The amount of Asp isomers in β- and γ-crystallin is smaller than in αA- or αB-crystallins.

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tallin-derived normal peptides and their isomeric peptides containing three different Asp isomers (L\(\beta\)-Asp-, D\(\alpha\)-Asp, and D\(\beta\)-Asp) and applied them to the LC-MS system. Fig. 4 shows the set of LC-MS chromatograms of \(\alpha\)A 55–65 (TVLDSGISEVR; \([M + 2H]^2+ = 588.3\) from the WI fraction (Fig. 4, a-1) and WS fraction (Fig. 4, a-2) of lens proteins from the 64-year-old donor and those of the synthetic peptides (Fig. 4, a-3). As shown in Fig. 4a-3, the TVLDSGISEVR peptides containing L\(\beta\)-Asp, D\(\alpha\)-Asp, D\(\beta\)-Asp, and D\(\beta\)-Asp eluted at 32.5, 37.2, 38, and 39.6 min, respectively. By comparison with the elution time of these synthetic peptides, peaks 1, 4, 5, and 7 in Fig. 4a-1 and a-2 were identified to be TVLDS(L\(\beta\)-Asp)SGISEVR, TVLDS(D\(\alpha\)-Asp)SGISEVR, TVLDS(D\(\beta\)-Asp)SGISEVR, and TVLDS(D\(\beta\)-Asp)SGISEVR peptides, respectively. Fig. 4, a-1 and a-2, show that the intensities of the isomeric peptides in the WI fraction are larger than those in the WS fraction. In the WI fraction, TVLDS(D\(\beta\)-Asp)SGISEVR is the highest peak, whereas in the WI fraction, TVLDS(D\(\beta\)-Asp)SGISEVR is the highest peak. Fig. 4b shows the set of LC-MS chromatograms of \(\alpha\)A 146–157 (IQTGLDATHAER; \([M + 2H]^2+ = 656.0\) from the WI fraction (Fig. 4, b-1) and the WS fraction (Fig. 4, b-2) of lens proteins from the 64-year-old donor and the synthetic peptides (Fig. 4, b-3). The synthetic IQTGLDATHAER peptides containing D\(\alpha\)-Asp, L\(\alpha\)-Asp, \(\beta\)-Asp, and D\(\beta\)-Asp residues eluted at 17.0, 17.5, 18.3, and 19.5 min, respectively (Fig. 4, b-3). Therefore, peaks 1, 2, 3, and 5 in Fig. 4, b-1 and b-2 were identified to be IQTGLD(D\(\alpha\)-Asp)ATHAER, IQTGLD(L\(\alpha\)-Asp)ATHAER, IQTGLD(\(\beta\)-Asp)ATHAER, and IQTGLD(D\(\beta\)-Asp)ATHAER by fitting with the retention time of the synthetic peptides (Fig. 4, b-3). Supplemental Fig. S1 shows the MS/MS spectrum of the synthetic peptides (TVLDSGISEVR and IQTGLDATHAER peptides) with the Asp isomers. Fig. 4, b-1 and b-2, indicate that the isomeric peptides occur more in the WI fraction than in the WS fraction. In the crystallin protein from the WI fraction, Asp isomers are clearly increased relative to those found in the WS protein.

Quantification of the Asp Isomers in Protein by LC-MS—To confirm whether there are differences between the intensities of the fragment ions of the L\(\alpha\)-Asp-containing peptide and those of the other three isomeric peptides, the four synthetic peptides were applied to LC-MS in various quantities. Fig. 5, a and b, show the relationship between the intensities of the diva-
lent ions and the amounts of the αA 55–65 peptide plus isomeric peptides and αA 146–157 peptide plus isomeric peptides, respectively. As shown in Fig. 5, a and b, there were no significant differences between the intensities of the divalent ions of the peptides and the isomeric peptides in either set of samples. The intensities of the divalent ions of αA 55–65 peptide including the isomeric peptides can be expressed as the linearity of the amounts of the sample peptides ranging from 1.0 to 1.0 in Fig. 5a.

Furthermore, we synthesized the following peptides and their three isomers: αA 79–88 (HFSPED(L-Asp)LTVK, HFSPED(D-Asp)LTVK, HFSPED(D-Asp)LTVK, and HFSPED(D-Asp)LTVK), αA 71–78 (FVIFLD(L-Asp)VK, FVIFLD(L-Asp)LTVK, FVIFLD(L-Asp)LTVK, and FVIFLD(L-Asp)LTVK), αB 57–69 (APSWFD(L-Asp)TGLEM, APSWFD(D-Asp)TGLEM, APSWFD(D-Asp)TGLEM, and APSWFD(D-Asp)TGLEM), and αB 93–103 (VLGD(L-Asp)VIEVHVK, VLGD(L-Asp)VIEVHVK, VLGD(L-Asp)VIEVHVK, and VLGD(L-Asp)VIEVHVK). These peptides also show the linearity between the intensities of the divalent ions and the amounts of the peptides at an intensity ranging from 1.0 to 1.0 in Fig. 5a. These results clearly indicate that the isomerization of Asp residues affects the higher order structure of crystallin through the inversion of the side chain to the D-form and through the extension of the backbone via formation of the β-linkage, which causes the crystallins in the soluble fraction to lose their solubility and move to the WI fraction of the lens.

**DISCUSSION**

Homochirality is essential for the development and maintenance of life. Until recently, the homochirality of proteins composed of L-amino acids was believed to be maintained, and the inversion of L-amino acids to D-amino acids was not thought to occur throughout the entire lifespan of an organism. However, D-amino acids were recently detected in various living higher organisms in the form of free amino acids, peptides, and proteins.

**TABLE 1**

| Name | Position | Peptide sequence | Fraction | 64Y | 74Y | 84Y |
|------|----------|------------------|----------|-----|-----|-----|
| αA   | 22-49    | LFDQFGGELFEDYLLPF | WI       | 0.30 | 0.23 | 0.24 |
|      |          | ESTSSPSYR        | WS       | 0.48 | 0.44 | 0.54 |
|      | 120-145  | LPSVGNLSCSLSDG   | MEITCQPK | 0.86 | 0.77 | 0.79 |
|      |          |                  | WS       | 0.97 | 0.88 | 0.81 |
|      | 1-11     | acMDVTIQHPWF     | WI       | 0.75 | 0.68 | 0.75 |
|      |          |                  | WS       | 0.95 | 0.86 | 0.93 |
|      | 146-157  | ITQGLDATHAER     | WI       | 0.38 | 0.37 | 0.33 |
|      |          |                  | WS       | 0.59 | 0.50 | 0.63 |
|      | 89-99    | QVDFVEIHLGK      | WI       | 0.11 | 0.38 | 0.39 |
|      |          |                  | WS       | 0.48 | 0.56 | 0.76 |
|      | 55-65    | TVLDSGISEVR       | WI       | 0.29 | 0.25 | 0.26 |
|      |          |                  | WS       | 0.64 | 0.51 | 0.59 |
|      | 79-88    | HFSPEDLTVK       | WI       | 0.79 | 0.78 | 0.82 |
|      |          |                  | WS       | 0.97 | 0.96 | 0.98 |
|      | 104-112  | QDQHGYSR         | WI       | 0.53 | 0.48 | 0.52 |
|      |          |                  | WS       | 0.70 | 0.73 | 0.60 |
|      | 71-78    | FVIFLDVVK        | WI       | 0.87 | 0.87 | 0.90 |
|      |          |                  | WS       | 0.96 | 0.96 | 0.98 |
|      | 124-149  | IPADVDPILHTSSLKASQTVNGPR | WI | 0.62 | 0.36 | 0.57 |
|      |          |                  | WS       | 0.74 | 0.40 | 0.62 |
|      | 57-69    | APSWFDTGLEM       | WI       | 0.41 | 0.36 | 0.47 |
|      |          |                  | WS       | 0.78 | 0.74 | 0.73 |
|      | 1-11     | acMDAALHPWIR     | WS       | 0.87 | 0.87 | 0.86 |
|      |          |                  | WS       | 0.99 | 0.93 | 0.95 |
|      | 93-103   | VLDGIVIEVHK       | WI       | 0.93 | 0.82 | 0.93 |
|      |          |                  | WS       | 0.99 | 0.95 | 0.95 |
|      | 108-116  | QDEHIHFIS         | WI       | 0.37 | 0.60 | 0.43 |
|      |          |                  | WS       | 0.80 | 0.71 | 0.74 |
|      | 75-82    | FSVNLDVK         | WI       | 0.90 | 0.93 | 0.90 |
|      |          |                  | WS       | 0.95 | 0.98 | 0.96 |
| αB   | 57-69    | APSWFD(L-Asp)TGLEM | WS       | 0.41 | 0.36 | 0.47 |
|      | 93-103   | VLDG(L-Asp)VIEVHK | WS       | 0.93 | 0.82 | 0.93 |
|      | 73-79    | WMGLNDR          | WI       | 0.82 | 0.64 | 0.92 |
|      | 149-154  | QYLLDK           | WI       | 0.86 | 1.00 | 0.92 |
|      |          |                  | WS       | 0.99 | 1.00 | 1.00 |
detected in various tissues such as teeth (29, 30), bone (31, 32), aorta (33), ligament (34), brain (35, 36), retina (37), conjunctiva (38), cornea (39), and skin (40, 41) of elderly individuals. It is therefore no longer uncommon to find d-amino acids in living organisms. The presence of d-aspartic acid in aged tissues of living organisms is thought to result from the racemization of the Asp residues in these particular proteins. Of all the naturally occurring amino acids, the Asp residue is the most susceptible to racemization. The racemization of Asp residues in proteins does not occur uniformly but does so at specific Asp residues on the basis of the sequence context or structural considerations that make the specific residues more susceptible to reaction than others. Therefore, it is necessary to determine the nature of the Asp residues at specific sites within particular proteins.

The separation of the optical isomers of amino acids has been considered to be difficult because the physical and chemical properties of the optical isomers are the same. Conventional enantioseparation of amino acids has been established by gas chromatography (GC) or RP-HPLC. The GC analysis involves direct enantioseparation through the use of a chiral capillary column, whereas RP-HPLC analysis involves indirect enantioseparation of the diastereoisomeric derivatives of the amino acid samples produced by chiral derivatizing reagents. Both methods require the appropriate amino acid derivatization or preparation in advance of the analysis; the former requires changing the samples into the gaseous state before injection for GC, and the latter requires production of diastereoisomeric derivatives in the case of the non-chiral column. The process for free d-amino acid analysis is very complex. In addition, to analyze the specific sites of d-amino acids in a protein, other steps more complicated than free d-amino acid analysis are required. These steps are as follows. (i) The protein is digested with an appropriate enzyme. (ii) The resulting peptides are separated by RP-HPLC. (iii) The peptides are identified by mass analysis and/or protein sequencing. (iv) The α- or β-isomer of the identified peptides is determined by Edman degradation reaction. (v) The D/L ratio of the identified peptides is determined after hydrolysis with 6 N HCl and derivatization. (vi) The diastereoisomers are analyzed by RP-HPLC, and the D/L ratio of amino acids is determined by analysis of the respective peak areas. The resulting analysis of the isomerization of Asp residues in a protein can be accurate, but it is a technically demanding process. Consequently, there has been little study of the presence and function of d-amino acids in living organisms.

FIGURE 4. LC-MS chromatograms of the tryptic peptides of αA 55–65 (TVLDSGISEVR) (a) and αA 146–157 (IQTGLDATHAER) (b) from the WI (a-1 and b-1) and WS fractions (a-2 and b-2) of 64-old-donor. a-3 and b-3 are LC-MS chromatograms of the synthetic peptides of αA 55–65 and αA 146–157, respectively.
A Rapid Survey of Asp Isomers in Aged Proteins

In the present study, we propose a new accurate and quick LC-MS-based analysis for determining the specific sites having Asp isomers and quantifying the amounts of Asp isomers at the individual sites of all lens crystallins in the WI and WS fractions without the need for complicated purification from the lens tissues. The isomeric peptides containing Asp isomers can be resolved because peptides with the same mass are separated into several peaks by LC-MS. Fig. 2, b and c, are typical LC-MS runs showing αA 55–65 and αB 57–69 separated into several peaks. These are expected to be the isomeric peptides resulting from isomerization of Asp-58 of αA-crystallin and Asp-62 of αB-crystallin with their amounts increased in the WI fraction over the WS fraction. However, there is still the possibility that different peptides with the same mass as αA 55–65 and αB 57–69 may be present in the profiles in Fig. 2, b and c. Therefore, to confirm the presence of the isomers, these peptides were analyzed by MS/MS analysis. The results clearly indicated that peaks 1–7 of Fig. 3a-A are all αA 55–65 peptide, whereas peaks 1–5 of Fig. 3b-B are αB 57–69 peptide, and peaks 6 and 7 are different peptides.

The detection of the isomeric Asp residues in the protein was achieved by the combination of finding peptides with the same mass that are separated into multiple peaks in LC-MS and their MS/MS analysis. Furthermore, we found isomeric peptides of all crystallins, that is αA-, αB-, βA3-, βA4-, βB1-, βB2-, and γS-crystallins, in both the WI and WS fractions of the lenses. The rI values are indicators of the presence of the isomeric peptides, and the smaller the rI value, the more isomers are present. The isomeric Asp residues are present in greater abundance in the WI fraction than in the WS fraction in all crystallins. The isomerization sites are Asp-24 and/or -35, -58, -91 and/or -92, -105 and/or -106, and -151 of αA-crystallin, whereas Asp-62 and -109 of αB-crystallin have a high isomeric percentage. The amounts of Asp isomers in β- and γ-crystallin are smaller than those in αA- or αB-crystallins. To determine which types of Asp isomers among δβ, δα, lβ, and lα are present, standard peptides were synthesized and applied to LC-MS. Fig. 4, a and b, show typical LC-MS chromatograms of αA 55–65 and αA 146–157 peptides, respectively. Peaks 1, 4, 5, and 7 in Fig. 4, a-1 and a-2, were identified to be the TVLD-(lβ-Asp)SGISEVR, TVLD(lα-Asp)SGISEVR, TVLD(δβ-Asp)-SGISEVR, and TVLD(δα-Asp)-SGISEVR by comparison with the elution time of the synthetic peptides. Peak 3 of Fig. 4a-1 was identified to be TVLD(lα-Asp)SGISEVR(lγ-Glu)VR by matching with the elution time of the synthetic peptides (supplemental Fig. S2). The other minor peaks may be the peptides containing the combination of Asp-58 isomers (δβ, lβ, and δα) and a γ-Glu residue at position 63. Peaks 1, 2, 3, and 5 in Fig. 4, b-1 and b-2, were identified to be IQTGLD(δα-Asp)ATHAER, IQTGLD(lα-Asp)ATHAER, IQTGLD(lβ-Asp)ATHAER, and IQTGLD(δβ-Asp)ATHAER. The intensities of the normal lα-Asp-containing peptides and the other three isomeric peptides containing δβ-Asp, lβ-Asp, and δα-Asp were the same in both peptides (Fig. 5). Therefore, it is possible for the ratio of the isomeric peptides to be calculated from the areas of LC-MS peaks. The most abundant isomers of Asp-58 and Asp-151 of αA-crystallin in the WS fraction were δβ-isomers, which is consistent with our previous results using conventional methods (4). The ratios of the δβ-Asp isomers to the normal lα-isomers at both Asp-58 and Asp-151 of αA-crystallin in the WI fraction are ~1:1 or greater.

Fig. 6 shows the relative amounts of the various Asp isomers of αA- and αB-crystallins from WI and WS fractions. The Asp-58 and Asp-151 of αA-crystallin and Asp-62 of αB-crystallin are highly inverted to the isomers in the WS fraction, which is entirely consistent with our previous results (4, 5), and their isomeric ratios increased dramatically in the WI fraction. In recent work, Hon et al. (42) reported that Asp-151 of αA-crystallin converted to δβ, lβ, and δα in a ratio of 3:1:0.5 until age 15 and that the percentage of δβ-Asp-151 is 40% by age 20 with a fixed ratio to age 80. These results are consistent with the present study. As shown in Fig. 1, the lα-Asp residue spontaneously converts to lβ-, δβ-, and δα-isomers in proteins through a succinimide intermediate. The rate of the succinimide formation depends on the neighboring residue of the Asp residue as well as the surrounding structure. Because the succinimide is unstable, it is hydrolyzed to form lβ-Asp or inverted to d-succinimide. If the deprotonation rate of l-succinimide is faster than the hydrolysis rate of l-succinimide, the amounts of d-succinimide should increase. In the case of crystallin, the deprotonation of l-succinimide may be predominant. It is well known that succinimide is hydrolyzed predominantly to the δβ form rather than the δα form (19). This is the reason why the δβ-Asp form is the most abundant in the lens crystallins.

The isomerization of Asp-76 and Asp-84 of αA-crystallin and Asp-96 of αB-crystallin was almost absent from the WS
fraction, but the isomers significantly increased in the WI fractions. The relative amounts of the isomers at Asp-76 and Asp-84 in the WI fraction were lower than the amounts of Asp-58 and Asp-151; however, the amounts of the isomers are very different between the WS and WI fractions. Therefore, it is proposed that the isomerization of Asp-76 and Asp-84 residues also contributes to the insolubilization and aggregation of $\alpha$-crystallin. In our previous study, we synthesized peptides corresponding to residues 70–88 (KFVIFLD\textsubscript{76}VKHFSPED\textsubscript{84}LTVK) of human $\alpha$-crystallin and its corresponding diastereoisomers in which L-$\alpha$-Asp was replaced with L-$\beta$-Asp, D-$\alpha$-Asp, and D-$\beta$-Asp at position 76 and compared their biochemical properties with that of the L-$\alpha$-Asp-containing peptide (43). The isomeric peptides containing L-$\beta$-Asp, D-$\alpha$-Asp, and D-$\beta$-Asp residues were more hydrophilic than the L-$\alpha$-Asp-containing peptide, and the isomeric peptides lost $\beta$-sheet structure and changed to random structures (43). The normal peptide promoted the aggregation of insulin, whereas the other three isomers suppressed the aggregation of insulin (43). Recently, Santhoshkumar \textit{et al.} (44) showed that the $\alpha$-$\alpha$-crystallin-derived peptide \textsubscript{66}SDRDKFVIFLDVKHF\textsubscript{80}, which accumulates in the aging lens, can inhibit the chaperone activity of $\alpha$-crystallin and can cause aggregation and precipitation of lens crystallins. The region including Asp-76 is located in the $\beta$-strand structure, and therefore the isomerization of the Asp may affect the secondary structure of the protein and induce insolubilization.

The isomerization of Asp in proteins is thought to occur when 1) the neighboring residue has a small side chain such as Gly, Ser, or Ala and 2) the Asp residues are present in flexible regions of the protein structure. The crystal structure of human $\alpha$-crystallin is not known, but that of bovine $\alpha$-crystallin has been reported (45). Because the conformations of bovine and human $\alpha$-crystallins are considered to be essentially similar due to the amino acid sequence similarity, it is possible to discuss the environment of the isomeric Asp residues in human $\alpha$-crystallin. Asp-84 and -151 are located in the unstructured region as shown in supplemental Fig. S3, and Asp-58 is easily accessible as a proteolytic target (46).

The lens crystallins are originally in the water-soluble fraction, but aggregation and insolubilization of the proteins proceed with age or cataract. The cause of the crystallin aggregation is not well understood; however, the increase in the Asp isomers may be one of the triggers for crystallin aggregation and insolubilization because the Asp isomers directly affect the protein structure via inversion of the side chain by the $d$-form and also via formation of the $\beta$-linkage. Consistent with this is that $\alpha$-$\alpha$-crystallin containing large amounts of $d$-$\beta$-Asp obtained from donors of 80 years has been shown to undergo abnormal aggregation to form massive and heterogeneous aggregates (18). In addition, the chaperone activity of aged $\alpha$-$\alpha$-crystallin was reduced by 60% relative to that of young $\alpha$-$\alpha$-crystallin (18). It is therefore necessary to determine the levels of isomeric aspartyl residues at specific sites in all crystallins from the WI and WS fractions of the lens.
Here we describe a convenient and robust biochemical method for identifying the isomeric Asp sites in crystallins using LC-MS systems. However, to determine which types of Asp isomers are present, synthetic peptides are required. With the exception of this point, there are many advantages to this new method: 1) no requirement for large amounts of sample proteins, 2) no requirement for the purification of lens proteins from W1 and W5 fractions, and 3) no requirement for complicated analytical steps, which usually include the hydrolysis of the peptides followed by derivatization to the diastereoisomers of amino acids. This new method is able to search comprehensively for the Asp isomers in damaged or aged proteins from all living tissues and cells. Furthermore, the isomeric Asp sites can be determined, and the amounts of the Asp isomers can be quantified quickly and accurately at the femtomole level. This new method therefore improves the study of the isomerization of any amino acid that occurs spontaneously in living tissues or cells.

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