Original Contribution

Simultaneous ultraviolet B-induced photo-oxidation of tryptophan/tyrosine and racemization of neighboring aspartyl residues in peptides

Simin Cai, Norihiko Fujii, Takeshi Saito, Noriko Fujii *
Research Reactor Institute, Kyoto University, Kumatori, Osaka 590-0494, Japan

A R T I C L E   I N F O

Article history:
Received 13 May 2013
Received in revised form
30 July 2013
Accepted 17 August 2013
Available online 30 August 2013

Keywords:
UV damage
Cataract
D-Amino acid
Photo-oxidation
Racemization
Free radicals

A B S T R A C T

Although proteins consist exclusively of L-amino acids, it is well known that D-isomers of aspartyl (Asp) residues occur at specific sites in lens crystallins of elderly people with cataracts. The presence of D-isomers is thought to result from the racemization of Asp residues in the crystallins during aging. It has been reported that this racemization progresses owing to UV-B exposure; however, the underlying mechanism remains unknown because Asp is not a photosensitive residue because there is no aromatic group in its chemical structure. In this study, we synthesized peptides in which the residue neighboring the Asp was the photosensitive residue tryptophan (Trp) or tyrosine (Tyr). After exposing these peptides to UV-B, we used RP-HPLC to confirm that racemization of Asp residues occurred in peptides in which a Trp or Tyr residue was inserted near the Asp; simultaneously, several varieties of photoproducts derived from Trp and Tyr were detected by mass spectroscopy. Promotion of the racemization of Asp residues in peptides with a neighboring Trp was much more significant than in those with Tyr. In particular, when Trp was next to an Asp residue on the C-terminal side of the peptide, the racemization reaction was accelerated.

The homochirality of proteins composed of L-amino acids was originally believed to be maintained throughout the entire life span of an organism. However, D-aspartyl (Asp) residues have been detected in various proteins from tissues of elderly individuals, such as lens [1–3], retina [4], conjunctiva [5], cornea [6], brain [7–10], skin [11,12], teeth [13–15], skeleton [16,17], and aorta [18]. Because these tissues are metabolically inert, the D-Asp residues arise from racemization of Asp residues in the protein during the life span of the individual. The accumulation of D-Asp might change the higher order structure of proteins and might decrease their function. Therefore, D-Asp formation and accumulation in human proteins are considered to be related to aging and disease [19]. We previously reported the presence of D-isomers at Asp-58, Asp-151 [1,3], Asp-76, and Asp-84 [3] in αα-crystallin; at Asp-36, Asp-62 [2,3], and Asp-96 [3] in ββ-crystallin; and at Asp-4 in ββ2-crystallin [20] from aged human lenses. D-Asp formation was accompanied by isomerization of the natural α-Asp to the abnormal β-Asp.

Racemization begins when the hydrogen atom attached to the α-carbon atom is released. Usually, this reaction proceeds with difficulty in mild conditions such as those found in the body. However, Asp residues in proteins are susceptible to racemization because Asp has a carboxyl group in its side chain. The mechanism underlying the inversion and isomerization of Asp residues in proteins is considered to proceed via a succinimide intermediate (Scheme 1). When the lone-pair electron of the nitrogen atom of the amino acid residue following the Asp residue attacks the carboxyl group of the side chain of the α-Asp residue, L-succinimide is generated by intramolecular cyclization. L-Succinimide can convert to D-succinimide via an intermediate through enol-keto tautomerism. Then, the D-succinimide is hydrolyzed at either side of its two carbonyl groups to form Dα- and Dβ-Asp; similarly, L-succinimide is hydrolyzed to form Lα- and Lβ-Asp. The rate of succinimide formation is considered to depend on the neighboring residue of the Asp residue. When the neighboring amino acid of the Asp residue has a small side chain, such as in alanine or serine, the formation of succinimide can occur easily because there is no steric hindrance [21,22]. On the other hand, D-isomers of Asp residues preceded by bulky leucine, threonine, or histidine residues have also been detected [2,20]. These results indicate that the formation of succinimide in proteins depends both on the neighboring amino acids of the Asp residues and on the higher order structure of the protein.

We have indicated that UV-B irradiation accelerates the racemization of Asp residues in proteins. UV-B irradiation induced Dβ-Asp formation at the Asp-151 residue in αα-crystallin and caused the opacity of lenses from 6-week-old rats [23]. Acceleration of the formation of D-Asp in proteins from aged human skin...
has also been demonstrated [11]. In the mouse skin, the formation of Dβ-Asp and advanced glycation end products, which are produced by the oxidation of glucose and the peroxidation of lipid, was simultaneously observed after UV-B irradiation [12]. The proteins were identified by proteomic analysis as members of the keratin families, including keratin-1, keratin-6B, keratin-10, and keratin-14 [12]. These results clearly indicated that UV irradiation accelerates the isomerization of Asp residues in proteins such as lens and skin, which are the tissues most susceptible to UV exposure from sunlight, and induces age-related diseases.

An Asp residue cannot absorb UV-B, however, so how is it possible that UV-B induces the racemization and isomerization of Asp residues in proteins? It is known that the most photosensitive residues of proteins are aromatic amino acids such as tryptophan (Trp) and tyrosine (Tyr) and sulfur-containing amino acids such as cysteine (because of the formation of disulfide bonds) [24]. The absorption of near UV by proteins depends mainly on Trp and Tyr (and to a very small extent on the amount of phenylalanine and disulfide bonds). In this report, the influence of neighboring photosensitive Trp or Tyr residues on the racemization of Asp residues in peptides in the presence of UV-B irradiation has been investigated. Here we show for the first time that Trp or Tyr residues accelerate racemization of Asp in the peptide.

**Materials and methods**

**Reagents**

Fmoc-amino acids, Fmoc-Arg (Mtr)-alko resin, HOBt- H2O, and PyBOP (Watanabe Chemical Industries); thioanisole, ethyl methyl sulfide, thiophenol, 2-methylindole, and trifluoroacetic acid (TFA) (Wako Pure Chemical Industries); N-methylmorpholine and 1,2-ethanediithiol (Nacalai Tesque); N,N-dimethylformamide and dimethyl ether (Sigma–Aldrich); and piperidine (Tokyo Chemical Industry) were used for solid-phase peptide synthesis.

Acetonitrile (AcN; Sigma–Aldrich) and TFA (Wako Pure Chemical Industries) acted as the mobile phase in reverse-phase high-performance liquid chromatography (RP-HPLC) for purification of synthesized peptides and purity detection.

Disodium phosphate and sodium phosphate were obtained from Wako Pure Chemical Industries for preparation of synthesized peptide solutions.

Neurotensin, α-cyano-4-hydroxycinnamic acid (CHCA; Sigma Chemical Co.), AcN (Sigma–Aldrich), and formic acid (FA), TFA, and acetonitrile (Wako Pure Chemical Industries) were used for mass spectroscopy.

Sodium hydroxide, methanol (MeOH), boric acid, o-phthalaldehyde (Wako Pure Chemical Industries); acetone, acetic acid, tetrahydrofuran (THF), AcN (Sigma–Aldrich); 6 N hydrochloric acid (HCl; Thermo Scientific); and N-tert-butoxycarbonyl-L-cysteine (Boc-L-Cys; Novabiochem) were utilized in the D/L ratio analysis of Asp residues.

**Solid-phase peptide synthesis**

The following seven peptides (a–g) were synthesized by a Shimadzu PSM-8 peptide synthesizer:

(a) IQtGldathaer (MW 1310.67), corresponding to residues 146–157 of the human eye lens α-crystallin A chain;
(b) IQtWldathaer (MW 1439.72), a peptide in which Gly was replaced with Trp at position 149 of peptide a;
(c) IQtCwdathaer (MW 1383.66), a peptide in which Leu was replaced with Trp at position 150 of peptide a;
(d) IQtGldwthaer (MW 1425.71), a peptide in which Ala was replaced with Trp at position 152 of peptide a;
(e) IQtGldawaer (MW 1395.70), a peptide in which Thr was replaced with Trp at position 153 of peptide a;
(f) IQtGydathaer (MW 1360.64), a peptide in which Leu was replaced with Tyr at position 150 of peptide a;
(g) IQtGldythaer (MW 1402.69), a peptide in which Ala was replaced with Tyr at position 152 of peptide a.

**Scheme 1.** A possible reaction pathway for spontaneous inversion and isomerization of Asp residues in proteins.
After the condensation reaction, 82.5% TFA, 5% H2O, 5% thioanisole, 3% ethyl methyl sulfide, 2.5% 1,2-ethanediethiol, and 2% thiophenol solution was added to peptides a, f, and g for cleavage of resin and peptide, as well as deprotection of the Fmoc group. For peptides b, c, d, and e, the cleavage solution consisted of 82.5% TFA, 5% H2O, 5% thioanisole, 3% ethyl methyl sulfide, 2.5% 1,2-ethanediethiol, 2% thiophenol, and 1 mg/ml 2-methylindole. The reaction time was controlled between 6 and 8 h. Dimethyl ether was added to the solution, followed by centrifugation at 3000 rpm for 5 min. The precipitate was then placed in a desiccator for 1 day.

Purification of synthesized peptides

Liquid chromatography–mass spectrometry (LC–MS; Thermo Scientific) was used to identify target peptides during the purification of the crude peptides by RP-HPLC (Tosoh). Peptide isolation was performed on a Capcell Pak C-18 type ACR column (10 × 250 mm; Shiseido). The following mobile-phase system was used: solution A (0.1% TFA/H2O, v/v) and solution B (0.1% TFA/AcN, v/v) at a flow rate of 3.0 ml/min. The eluent was monitored at 230 (peptide a) and 280 nm (peptides b–g). The analysis was conducted with a mobile-phase gradient of 15–40% solution B over 60 min. The target purified peptide solutions were frozen at −80 °C and vacuum dried in a freeze dryer.

Measurement of purity of purified peptides

Purified peptide solutions were analyzed by RP-HPLC (Jasco) to measure their purity. The analyses were performed on a Capcell Pak C-18 type UG80 column (3.0 × 250 mm; Shiseido). The following mobile-phase system was used: solution A (0.1% TFA/H2O, v/v) and solution B (0.1% TFA/AcN, v/v) at a flow rate of 0.5 ml/min. The eluent was monitored at 215 nm. The purity analysis of peptides was conducted with a mobile-phase gradient of 60 min (0–50%, solution B). All synthesized peptides in this work were confirmed to be more than 95% pure.

UV-B irradiation of synthesized peptides

Three hundred microliters of 0.1 mg/ml peptides a–g and 50 mM phosphate buffer (pH 7.0) were placed into quartz cells (type S10-UV-1, 1 × 10 × 45 mm). Peptides a–e were irradiated at a dose rate of 0.2 mW/cm² of UV-B with a UV-B medical light source (Philips PL-S 9W/12/2P) measured by a UV radiometer (UVP E27530 Model UVX-31). One-hundred-microliter aliquots of peptides a–e in solution was removed after 17, 35, and 69 J/cm² UV-B irradiation. Peptides a, f, and g were irradiated at a 10 mW/cm² dose rate of UV-B. One-hundred-microliter aliquots of peptides a, f, and g in solution were collected after 86, 173, and 346 J/cm² UV-B irradiation. For each peptide, three parallel experiments were carried out on separate days. The experimental conditions for UV-B irradiation were determined on the basis of the following estimation: when calculating the energy emitted by UV-B that reaches the surface of earth, the energy absorbed by the lens over one summer is estimated to be about 270 J/cm². Because lens crystallin is known to block about 30% of UV-B, the energy absorbed by the lens over one summer is approximately 80 J/cm². According to this value, we selected the dose of 69 J/cm² of irradiation (to tryptophan-containing peptides). For peptides within tyrosine, because the absorption of UV-B of tyrosine is much lower than that of tryptophan, we raised the dose to 5 × 69 J/cm².

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) performed for photo-oxidation detection

UV-B-treated 0.1 mg/ml peptides a–e (35 J/cm² irradiated) in 50 mM phosphate buffer (pH 7.0) and 0.1 mg/ml peptides a, f, and g (173 J/cm² irradiated) in 50 mM phosphate buffer (pH 7.0) were desalted using the following steps to be analyzed by MALDI-TOF-MS (AXIMA-TOF²; Shimadzu): isometric 2.5% TFA was added to each peptide sample, and 1 μl of diluted sample was dripped onto the target plate after drying of 20 mg/ml CHCA/acetone. After 3 min, the drop on the target plate was soaked up using a KimWipe. Then 1 μl of 0.1% TFA was dripped onto the dry sample and soaked up after 30 s. Nonirradiated peptide a–g solutions were desalted and used as negative controls. CHCA in acetone solution (20 mg/ml) was used as a matrix; 0.01 mg/ml neurotensin was used as a standard for calibration.

LC–MS for analysis of photo-oxidized amino acids

UV-B-treated 1 μg/ml peptide c (35 J/cm² irradiated) was analyzed by LC–MS (Thermo Scientific). Untreated peptide c was applied and used as a negative control. Peptide isolation was performed by liquid chromatography combined with mass spectrometry on an L-column Micro column (0.1 × 150 mm; CERI). The following mobile-phase system was used: solution A (0 (1.0% FA, 2% AcN/H2O, v/v) and solution B (0.1% FA, 90% AcN/H2O, v/v) at a flow rate of 0.5 μl/min. The isolation of photoproducts was performed with a mobile-phase gradient of 5–45% solution B over 0–60 min.

Acid hydrolysis of UV-B-irradiated peptides

UV-B-irradiated peptides a–g were hydrolyzed in gas-phase 6 N HCl in vacuo at 108 °C for 7 h on a Pico Tag work station (PTS-3157; Waters). Nonirradiated peptides were hydrolyzed and used as a negative control. The hydrolyzed samples were redissolved in 30 μl of Milli-Q water after HCl gas was delivered; then 15 μl of each sample was diluted by addition of 60 μl of 0.13 M borate buffer (pH 10.4). Boc-L-Cys/MeOH (111 mg/ml) and 5 mg/ml o-phenyaldehyde/hyde/MeOH were added to produce fluorescent diastereoisomers.

RP-HPLC of diastereoisomers derived from acid hydrolysates

Acid hydrolysates were analyzed by HPLC (Shimadzu), performed on a Nova-Pak ODS column (3.9 × 300 mm; Waters) using fluorescence detection (344 nm as excitation and 433 nm as emission wavelength) [25]. The following mobile-phase system was used: solution A (5% AcN, 3% THF/0.1 M acetate buffer, pH 6.0) and solution B (47% AcN, 3% THF/0.1 M acetate buffer, pH 6.0) at a flow rate of 0.8 ml/min. The diastereoisomers were eluted with a mobile-phase gradient as follows: 0–7 min, 0–1% solution B; 7–10 min, 1–3% solution B; 10–21 min, 3–6% solution B; and 21–28 min, 6–55% solution B. i-Cys-Asp diastereoisomers were identified by their retention times (i-Cys-i-Asp, 15.9 min; i-Cys-o-Asp, 17.8 min). Peaks were quantified via standard curves constructed using authentic materials.

Results

Mass spectra of nonirradiated and 35 J/cm²-irradiated peptides a–e

With both nonirradiated and UV-B-irradiated peptide a (IQTGLDATHAER), essentially identical spectra were obtained with the precursor ion ([M + H]+ = 1311 m/z) and Δm/z = +22 ion (1333 m/z), which was considered to be an [M + Na]+ ion (Fig. 1).
However, for peptides b (IQTWLDATHAER), c (IQTGWLDATHAER), and e (IQTGLDATHAER), after UV-B irradiation, not only were precursor ions detected, but also Δm/z = +4, +16, +32, +46, +86, or +109 ions were observed. In addition, Δm/z = +16, +32, and +46 ions were detected the most frequently among the above ions (Figs. 2–5). These results indicate that after UV-B irradiation, peptides b, c, d, and e—that is, the peptides that have a Trp residue in their sequence—were photo-oxidized at some location(s).

Mass spectra of nonirradiated and 173 J/cm²-irradiated peptides a, f, and g

In both the 35 J/cm²-irradiated peptide a (IQTGLDATHAER) and the 173 J/cm²-irradiated peptide a, only the precursor ion (1311 m/z) and [M+Na]⁺ ion (1333 m/z) were detected (Fig. 6). However, for peptides f (IQTGYDATHAER) and g (IQTGLDYTHAER), after UV-B irradiation, not only the precursor ions, but also the Δm/z = +14, +30, or +32 ions were detected (Figs. 7 and 8). These results indicate that after UV-B irradiation, in addition to peptides b–e, peptides f and g were photo-oxidized at some location(s). However, the amounts of oxides of peptides f and g were smaller than those of peptides b–e when comparing the signal intensity.

Furthermore, the mass spectra of 173 J/cm²-irradiated peptides f and g showed a great deal of smaller m/z value peaks (Figs. 7 and 8); these peaks were more obvious than in the case of the 35 J/cm²-irradiated peptides b–e (Figs. 2–5). For the 173 J/cm²-irradiated peptide a, however, this phenomenon was not observed (Fig. 6). These results indicate that after relatively high doses of irradiation (173 J/cm²), peptides containing amino acid residues that can absorb UV-B are easily broken into small fragments.

LC–MS/MS spectra of peptide c

UV-B-irradiated peptide c was used as an example to ascertain the position of the Δm/z = +16 oxidized amino acid residue by LC–MS/MS. Fig. 9 shows the LC–MS/MS fragment spectra of nonirradiated peptide c with m/z 1384 (Fig. 9, top) and 35 J/cm² UV-B-irradiated peptide c with m/z 1400 (Fig. 9, bottom). It is clear that the b₄ ion of peptide c in both traces is 400.2 m/z, whereas the b₅ ion has different values, with m/z of 586.3 (top trace) and 602.3 (bottom trace). This result indicates that the b₅ ion in Fig. 9, bottom, has a mass 16 units bigger than that in Fig. 9, top. As well as b ions, the y₇ ion in both traces has the same value of 799.4 m/z, yet the y₈ ion in Fig. 9, bottom (1001.4 m/z), has a mass 16 units bigger than that in the top trace (985.4 m/z). These results demonstrate that in UV-B-irradiated peptide c, it is the Trp residue that is photo-oxidized with Δm/z = +16.

According to this result, the photo-oxidation in peptides b–g was considered to occur at the Trp/Tyr residue. The photo-oxides detected in these peptides were presumed to be the following: in the case of Trp, the most abundant Δm/z = +16 and +32 ions were proposed to be hydroxytryptophan/oxindolyalanine (W+O) and N-formylkynurenine/dihydroxytryptophan, respectively; the
other ions detected with $\Delta m/z = +4$, $+48$, and $+64$ ions were proposed to be kynurenine ($W + O - C$), hydroxyformylkynurenine ($W + 30$), and dihydroxyformylkynurenine ($W + 40$), respectively; in the case of Tyr, the $\Delta m/z = +14$, $+30$, and $+32$ ions were proposed to be dopa-quinone ($Y + O - 2H$), topa-quinone ($Y + 2O - 2H$), and topa ($Y + 2O$). These proposals are also consistent with the results reported by Grosvenor et al. [26]. However, in peptides containing a Trp residue, a large amount of the $\Delta m/z = +46$ ion was detected, but it was not clear whether this was a type (or types) of Trp photo-oxide.

**Fig. 3.** Mass spectra of peptide c (IQTWGDATHAER). (Trace a) Nonirradiated; (trace b) 35 J/cm² irradiated.

**Fig. 4.** Mass spectra of peptide d (IQTGWDTWAHER). (Trace a) Nonirradiated; (trace b) 35 J/cm² irradiated.

**Fig. 5.** Mass spectra of peptide e (IQTGDAWHAER). (Trace a) Nonirradiated; (trace b) 35 J/cm² irradiated.

Table 1 shows the $\nu/l$ ratios of Asp in peptides irradiated at 0–69 J/cm² and the growth rate of the $\nu/l$ ratios by comparing...
nonirradiated peptides and peptides after 69 J/cm² irradiation. The growth rate was estimated by the following equation:

\[
\text{growth rate (\%)} = \frac{[(69 \text{ J/cm}^2\text{-irradiated D/L ratio)} - \text{nonirradiated D/L ratio}]}{\text{C}^2} \times 100.
\]

Fig. 10 shows the histogram of Asp D/L ratio changes in peptides a–e in relation to UV-B doses and the results of statistical analysis. Differences in the Asp D/L ratio between the irradiated (17, 35, or 69 J/cm², peptides a–e) and the nonirradiated peptides were analyzed using Student’s t test. When the P value was less than 0.05, the difference between the two groups of data was significant; when the P value was less than 0.01, the difference was greatly significant; when the P value was more than 0.05, the difference was not significant.

The Asp D/L ratio of peptide a (IQTGLDAHTHER), which contained Asp but no aromatic residue in the peptide sequence, did not increase after 69 J/cm² irradiation. However, when the Ala residue in peptide a was replaced by Trp (peptide d; IQTGLDATHAER), the racemization of Asp dramatically increased in a UV-B-dose-dependent manner and the growth rate rose by 76% after 69 J/cm² irradiation (Fig. 10a, Table 1). Comparing the
Asp D/L ratio growth rate between peptides c (IQTGWDATHAER) and d (IQTGLDATHAER), it was clear that the Asp D/L ratio of peptide d increased faster than that of peptide c (Fig. 10b). The increase in the Asp D/L ratio in peptide d was significant after irradiation with 17 J/cm² and it depended on UV-B dose; however, for peptide c, the increase in the Asp D/L ratio was significant only after irradiation with 35 J/cm², although it still showed a UV-B dose dependency. The growth rate of peptides c and d after 69 J/cm² irradiation was 42 and 76%, respectively (Fig. 10b, Table 1). This result indicates that when Trp is on the neighboring C-terminal side of Asp, racemization is promoted more than when it is on the neighboring N-terminal side. For peptides d and e (IQTGLDASHAER), which had Trp on the C-terminal side of Asp, the Asp D/L ratio of peptide d grew significantly faster than that of peptide e, in which the Trp was separated from the Asp by one residue. Comparing the statistical significance of the data, the increase in Asp D/L ratio for peptide e was significant after a dose of 35 J/cm² and was dependent on UV-B dose; the increase in Asp D/L ratio for peptide d was significant after a dose of only 17 J/cm² and was also dependent on UV-B dose (Fig. 10c). However, there was no significant difference between peptides b (IQTWLDATHAER) and c (Fig. 10d). These results suggest that when Trp is on the C-terminal side of Asp in the peptide sequence, the distance between Trp and Asp affects racemization—in other words, the shorter the distance is, the faster the reaction occurs. However, when Trp is on the N-terminal side of Asp, the effect on racemization is not obviously changed by shortening the distance between Trp and Asp in the peptide sequence. Notably, the racemization of Asp was increased by UV-B exposure in a dose-dependent manner when Trp was followed by Asp in the peptide sequence.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Dose (J/cm²)</th>
<th>Growth rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>a (IQTGLDATHAER)</td>
<td>0.041</td>
<td>0.041</td>
</tr>
<tr>
<td>b (IQTWLDATHAER)</td>
<td>0.046</td>
<td>0.051</td>
</tr>
<tr>
<td>c (IQTWDATHAER)</td>
<td>0.033</td>
<td>0.035</td>
</tr>
<tr>
<td>d (IQTGLDWHAAER)</td>
<td>0.013</td>
<td>0.049</td>
</tr>
<tr>
<td>e (IQTGLDAWHAA)</td>
<td>0.037</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Table 2 shows the D/L ratios of Asp in the peptides irradiated at 86–346 J/cm² and the growth rate at 346 J/cm² of UV-B. The growth rate was estimated using the following equation:

\[
\text{growth rate (\%) } = \frac{[(346 \text{ J/cm}^2 - \text{irradiated Asp D/L ratio}) - \text{nonirradiated Asp D/L ratio}]}{\text{nonirradiated Asp D/L ratio}} 	imes 100.
\]

Fig. 11 shows the histogram of Asp D/L ratio changes for peptides a, f, and g in relation to UV-B doses and the results of statistical analysis. Differences in the Asp D/L ratio between the irradiated (86, 173, or 346 J/cm²) and the nonirradiated peptides were analyzed using Student’s t test. The Asp D/L ratio of peptide a (IQTGLDATHAER) was barely increased after irradiation with a dose of 346 J/cm². However, for
peptides f\(\text{(IQTGYDATHAER)}\) and g\(\text{(IQTGLDYTHAER)}\), which had a Tyr residue at the site neighboring the Asp, the Asp D/L ratio had significantly increased after a dose of 86 J/cm\(^2\), and the increase was dependent on the UV-B dose (Table 2, Fig. 11). The growth rate of both peptides f and g was about 70%, although the irradiation UV-B doses (346 J/cm\(^2\)) were five times larger than those used for peptide c (69 J/cm\(^2\)). This result indicates that the effect on Asp racemization of the Tyr residue is much less than that of the Trp residue.

In contrast to the significant difference in growth rate observed for peptides c\(\text{(IQTGWDATHAER)}\) and d\(\text{(IQTGLDWTHAER)}\), the Asp D/L ratios of peptides f and g were almost the same (Fig. 11). This indicates that changing the position of the Tyr residue from N- to C-terminal of Asp has little effect on its racemization.

Discussion

It is well known that overexposure to UV-B radiation can cause sunburn and some forms of skin cancer. Our recent study clearly indicated that UV-B irradiation enhances ω-Asp generation in keratin-1, -6B, -10, and -14 of epidermis. The amounts of D-Asp in these proteins increased in a UV-B-dose-dependent manner [12]. High intensities of UV-B light are also hazardous to the eyes, and exposure can cause photokeratitis and may lead to cataracts, pterygium [27,28], and pinguecula formation. In previous studies, it was reported that ω-Asp was found in both aged and UV-B-irradiated lens proteins. However, the relationship between the generation of ω-Asp and UV-B irradiation in not well understood, because the Asp residue cannot absorb UV-B because there is no aromatic group in its chemical structure.

In the present study, it has been demonstrated that ω-Asp can be generated by UV-B exposure when there is a Trp or Tyr near the Asp residue in the peptide sequence. According to this result, we...
propose that during the process of UV-B irradiation, the energy of the UV-B is absorbed by the aromatic amino acid (Trp or Tyr), which might lead to simultaneous photo-oxidation of Trp/Tyr and racemization of the nearby Asp residue. After UV-B irradiation, peptides containing Trp/Tyr were photo-oxidized at the Trp/Tyr position. Several kinds of products were detected by mass spectroscopy. Because the UV-B absorption of Trp is much higher than that of Tyr, Trp could be photo-oxidized more easily after relatively low doses of irradiation. Relatively high doses of UV-B irradiation led to serious damage to peptide sequences containing amino acid residues that can absorb UV-B.

In peptides that had Trp near the Asp, isomerization increased much more significantly than in the Tyr-containing peptides. In particular, when Trp was on the C-terminal side of the Asp residue, the increase in racemization was clearly increased by shortening of the distance between the Trp and the Asp residues. The reason for this phenomenon is proposed to be a result of the possible mechanism of isomerization of Asp (Scheme 1), in which generation of \( \alpha \)-succinimide occurs on the nearby in terms of the peptide sequence, the D/L ratio increased to 6-week-old rats. Although these Asp residues have no Trp residue, the highly inverted Asp-151 residue is located close to the Trp-9 residue in the peptide sequence. Therefore, the reaction might be affected more significantly when Trp is on the C-terminal side of the Asp residue. However, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In peptides that had Trp near the Asp, isomerization increased much more significantly than in the Tyr-containing peptides. In particular, when Trp was on the C-terminal side of the Asp residue, the increase in racemization was clearly increased by shortening of the distance between the Trp and the Asp residues. The reason for this phenomenon is proposed to be a result of the possible mechanism of isomerization of Asp (Scheme 1), in which generation of \( \alpha \)-succinimide occurs on the nearby in terms of the peptide sequence, the D/L ratio increased to 6-week-old rats. Although these Asp residues have no Trp residue, the highly inverted Asp-151 residue is located close to the Trp-9 residue in the peptide sequence. Therefore, the reaction might be affected more significantly when Trp is on the C-terminal side of the Asp residue. However, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.

In our previous study, the \( \omega \)-isomer of Asp-97 (WD\( \alpha \)WGS-NAYHER) in aged human lens \( \beta \)A3-crystallin was detected [3]. Racemization of Asp-97 might be promoted by Trp residues near the Asp residue in the peptide sequence, consistent with the results of this work. In addition, we previously indicated that UV-B irradiation induced the opacity of lenses and accelerated \( \rho \)-\( \beta \)-Asp formation at the Asp-151 residue in \( \alpha \)A-crystallin in 6-week-old rats. Although these Asp residues have no Trp residue, the position of Tyr hardly affects the racemization of Asp because the absorption of UV-B by Tyr is much lower than that by Trp.


