The Degradation and Regeneration of α-Lipoic Acid under the Irradiation of UV Light in the Existence of Homocysteine

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Summary α-Lipoic acid (LA) is the one of the strongest antioxidants to be utilized in supplement, skin ointment and so on. The distorted five membered dithiolane ring of LA, which is necessary structure to work as a cofactor of enzyme, is considerably vulnerable to UV irradiation. LA is easily decomposed by photoirradiation resulting in the loss of its characteristic absorption band at 333 nm. The photodegradation of LA means loss of its physiological activity, so that protection of LA from UV light is eagerly desired. Thiol compounds can be regarded as a potential candidate. In order to pursue the possibility of the thiol compounds in prevention of LA degradation, we examined the photoirradiation of LA in the presence and absence of homocysteine.

Key Words: α-lipoic acid, UV degradation, biothiol

Introduction

α-Lipoic acid (racemate, LA) is one of the attracted antioxidants [1, 2], which is applied in the usage of skin ointment to protect human skin from the damage by ultraviolet (UV) light [3]. Although the protective effect of LA against UV induced skin damage is absolutely doubtless [4, 5], the degeneration of LA by UV light is just about to be regarded as a hidden problem [6, 7]. LA has moderate absorption band at 333 nm due to the hindered dithiolane ring, which is easily decomposed by the photoirradiation. Matsugo et al. [8] reported the degradation of LA and simultaneous generation of dihydrolipoic acid (DHLA) by the UVA irradiation. They proposed the formation of dithiyl radical by the rupture of disulfide (S-S) bond followed by the intra or intermolecular hydrogen atom abstraction to form DHLA. Recently, Bucher et al. reported the further investigation on the decomposition mechanism by using the combination of laser flash photolysis and computational calculations [9]. They revealed not only the existence of dithiyl radical but also other activated species such as carbon-centered radicals caused by the cleavage of C-S bond or intramolecular hydrogen abstraction of dithiyl radical. They also reported unique results such that LA was regenerated from DHLA by the irradiation of UVC light (265 nm). Their results indicate that decomposition and regeneration of LA should occur simultaneously under UV light. Inhibition of side reactions involved in the initial stage of photoirradiation, which associated with radical reaction, is not only the key factor to accomplish selective ring opening and closing but also one of the approaches for the protection of LA from degradation. The radical transfer reagent such as thiol compound is the possible candidate to protect LA from decomposition. Thiol compounds were widely used as a radical transfer reagent in the polymer synthesis [10]. In order to pursue the possibility of the thiol compounds in the prevention of LA degradation, we examined the photoirradiation of LA in the presence and absence of homocysteine.
Experimental

General

LA (racemate) and ethyl acetate (AcOEt) was purchased from Kanto Chemical Co. (Tokyo, Japan). Ethanol (EtOH), methanol (HPLC grade, MeOH), chloroform (CHCl₃), sodium sulfate (Na₂SO₄), DL-homocysteine (Hcy) and di-Sodium hydrogenphosphate (Na₂HPO₄) were purchased from Nacalai Tesque Co. (Kyoto, Japan). Ultrapure water was prepared by using ADVANTEC PWU-100. UV-vis absorption spectra were measured by using JASCO V-550. HPLC analysis was carried out on Hewlett Packard HPLC Series 1100 system with a column of COSMOSIL PACKED COLUMN 5C18-MS. The samples were eluted with phosphate buffer (pH 7.4) and MeOH (buffer/MeOH = 4/6, 0.7 mL/min) and detected at 333 nm. ¹H-NMR spectra were measured in CDCl₃ at 20°C by using JEOL JNM-GSX 500 and chemical shifts were expressed relative to Me₄Si (TMS) as an internal standard. The calibration curves of LA and DHLA was made, which has the axes of the concentration and integral ratio against TMS. The integral ratios of the signals at 3.17, 3.58 ppm for LA and 1.30, 1.35 ppm for DHLA were used to make calibration curves. The amount of LA and DHLA in sample solution was estimated by the integral ratios of the relevant signals against TMS. In all measurements, the integral value of TMS was fixed at 1 and the volume of CDCl₃ was 0.6 mL. UV irradiation was performed on NTM-10 transilluminator (Funakoshi, Tokyo, Japan) using a cut-off filter of 302 nm. Stock solution (5 and 10 mM) of LA in 10 mM Na₂HPO₄ solution was prepared and LA concentration of all samples for UV irradiation was fixed at 5 mM.

Reduction of LA to DHLA

DHLA was synthesized as a standard sample to make a ¹H-NMR calibration curve. LA (206 mg, 1.0 mmol) was dissolved in EtOH (10 mL, N₂ purged) under N₂ atmosphere at 0°C. NaBH₄ (151 mg, 4.0 mmol) was added under N₂ atmosphere at 0°C and the reaction mixture was stirred for 30 min at room temperature (r.t.) followed by the acidification by HCl aq. (10 v%). The crude mixture was extracted with CHCl₃ and the filtrate was dried in vacuo to afford DHLA (207 mg, 0.99 mmol, 99%) as clear colorless liquid.

Determining the irradiation time

5 mL of LA solution (5 mM) in a glass vial placed on the transilluminator was irradiated for 60 min at r.t. The absorbance at 333 nm was monitored by every 10 min during the irradiation.

LA behavior without homocysteine

5 mL of LA solution (5 mM) in a glass vial placed on the transilluminator was irradiated at r.t. under aerobic conditions for 30 min. After the UV irradiation, the solution was kept at r.t. under aerobic conditions for 15 h. The UV-vis absorption
Fig. 2. $^1$H-NMR spectra of the extracts (a) just after the irradiation and (b) kept for 15 h under air (CDCl$_3$, 20°C). Assignment of the signals of LA and DHLA were also represented. The up- and downward-arrows mean the increase and decrease of the relevant signal compared to that of spectrum (a). $^1$H-NMR spectra around the signals of (e), (g) and (o), (p) were enlarged in the inset.

absorption spectra and $^1$H-NMR spectra of the sample were measured just after the irradiation and after 15 h standing. The samples for $^1$H-NMR measurements were prepared by extracting reaction mixtures with CHCl$_3$ (3, 2 and 2 mL) until the absorbance of water layer did not change. The gathered CHCl$_3$ solution was removed in vacuo and the residue was subjected to the $^1$H-NMR analysis.

LA behavior with homocysteine

5 mL of LA solution (10 mM) and adequate volume of Hcy solution (50 mM) was mixed in a glass vial followed by diluting with 10 mM Na$_2$HPO$_4$ to 10 mL. The samples were prepared as final Hcy concentrations at 0, 5 and 25 mM, respectively. These vials were irradiated from the bottom of the vials for 30 min and stood for 15 h at r.t. The UV-vis absorption spectra, HPLC analysis and $^1$H-NMR spectra of the sample solution were measured just after the irradiation and after standing for 15 h. The samples for $^1$H-NMR measurements were prepared by extracting reaction mixtures (LA: 5 mM, Hcy: 25 mM) with CHCl$_3$. The extraction and $^1$H-NMR measurements procedures were the same as described above.

LA behavior by the addition of homocysteine after UV irradiation

5 mL of LA solution (5 mM) in a vial with a plastic cap was irradiated from the bottom of the vial for 30 min at r.t. After the UV irradiation, Hcy were added (0, 5 and 25 mM) and the reaction mixtures were stood for 15 h at r.t. under aerobic condition. UV-vis absorption spectra were measured just after the irradiation (before adding Hcy) and after the standing for 15 h.

Results

UV-vis absorption spectral analysis

According to the results of UV-vis absorption spectra, the specific absorption band due to the dithiolane ring of LA
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was found to decrease time-dependently by UV irradiation using a transilluminator (cutoff 302 nm) and reached a stationary state after 30 min irradiation. After standing for 15 h after UV irradiation, the absorbance at 333 nm was slightly recovered [For example, compare Fig. 1 (b)-(i)-(II), with Fig. 1 (b)-(i)-(III)]. When LA was irradiated in the presence of Hcy, absorbance decrease at 333 nm was significantly inhibited depending on the concentration of Hcy (Fig. 1 (b)-(II)). In this case, additional absorption band was detected around at 375 nm, which overlaps the absorption band edge of LA (Fig. 1 (a)). This problem made it difficult to estimate the exact amount of LA. After keeping the solution for 15 h, the band at 375 nm was disappeared and absorbance of LA was recovered dependent on Hcy concentration (Fig. 1 (b)-(III)). When Hcy was added after the irradiation, the recovered amount of LA was increased depending on the concentration of Hcy (Fig. 1 (c)-(II), (III)).

HPLC analysis

HPLC analysis was performed to overcome the problem of UV-vis absorption spectral analysis. The peak area of LA with retention time at 3.3 min decreased just after the irradiation but recovered after 15 h standing. At the same time, we found four kinds of by-products were formed during the irradiation.

1H-NMR spectral analysis

LA solution just after the irradiation and after standing for 15 h was extracted with CHCl₃, and was subjected to 1H-NMR measurements. Based on the calibration curve obtained by 1H-NMR studies described in the experimental section, we calculated the LA remained to be 14% and DHLA to be 12% without Hcy (Fig. 2 (a)). The amount of LA was increased to 25%, while DHLA was reduced to almost 2% after 15 h (Fig. 2 (b)). The increase and decrease

Fig. 3. 1H-NMR spectra of the extracts (a) just after the irradiation with Hcy (25 mM) and (b) kept for 15 h under air (CDCl₃, 20°C). Assignment of the signals of LA and DHLA were also represented. The up- and downward-arrows mean the increase and decrease of the relevant signal compared to that of spectrum (a). 1H-NMR spectra around the signals of (e), (g) and (o), (p) were enlarged in the inset.
of signals were indicated by up- and downward-arrows, respectively. When Hcy (25mM) was present under the irradiation, the decomposition of LA was slightly prevented (25%) and DHLA was formed preferentially (44%) (Fig. 3 (a)). The amount of LA was thus increased to 51% and DHLA was reduced to 18% after 15 h (Fig. 3 (b)).

Discussion

All experiments support the protective effect of Hcy on the degradation of LA under UV irradiation (by a transluminator using cutoff filer 302 nm). Although UV-vis absorption spectrum indicated the photodecomposition and regeneration of LA, quantitative discussion could not be performed due to the presence of the absorption band at 375 nm as initial products. On the contrary, the 1H-NMR measurements were only carried out about the samples of CHCl3 extracts, so that some of the by-products in water layer were not detected. The 1H-NMR results somewhat underestimated the total amount of LA and DHLA because 1H-NMR measurements were carried out only using CHCl3 extracts (not 100%). These are considerable reasons of the discrepancy between the results of UV-vis absorption spectra and 1H-NMR spectra. There are some limitations of 1H-NMR estimation: nevertheless, 1H-NMR spectral analysis is absolutely one of the useful methods to determine both chemical structure and the amount. According to the results of 1H-NMR measurements, the additive of Hcy induced to generate DHLA under the UV irradiation, which resulted in the improved recovery of LA. The complicated 1H-NMR spectra in Fig. 2 than those of Fig. 3 indicate that Hcy can suppress the generation of hydrophobic by-products. The increased amount of LA was found to be 11% to 26% in the coexistence of Hcy under the irradiation. The 15% of improvement was predominantly derived from the selective conversion of LA to DHLA. The development of DHLA generation indicates the mechanism through the rupture of disulfide bond. Two possible mechanisms for selective conversion can be proposed. One is the direct hydrogen abstraction of dithyl radical from Hcy, which also contribute to prevent the generation of reactive oxygen species (ROS). ROS can be produced by the reaction between dithyl radical and oxygen in water. The other is the antioxidant ability of Hcy. To avoid the reaction of DHLA with ROS, Hcy might play a role in inhibiting ROS. This anticipation, however, does not agree with the result of ascorbic acid, in which ascorbic acid did not improve the generation of thiol group on the irradiation of LA [8]. Further investigation is necessary to clarify the mechanism. The results in this study suggest that other biothiols can also contribute selective conversion and protection of LA. The effect of other biothiols is now under investigation.

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