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SDS-PAGE study on photooxidation damage of lysozyme induced by riboflavin

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The photooxidation damage of lysozyme under 315—375 nm irradiation in the presence of riboflavin was studied by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Indications showed that the mechanisms and products of oxidative damage were relative to the concentration of riboflavin, the time of irradiation and the ambience. The type I process was examined in a nitrogen saturated solution, whereas both type I and type II were observed in an aerobic atmosphere and type II was the dominant process. The study also suggested that antioxidants, such as melatonin, can reduce the damage of lysozyme effectively.

lysozyme, riboflavin, photooxidation, SDS-PAGE, antioxidant

Riboflavin (RF), commonly known as vitamin B_2, is an essential component of living organisms and widely exists in aerobic cells. Human can ingest RF from milk, beer, yeast and leafy vegetables. RF possesses intensive absorption in UVB (290—320 nm), UVA (320—380 nm) and the visible light region, in Figure 1(a). RF can be excited under UV irradiation, and the excited singlet state ($^1RF^*$) hardly reacts with other materials due to its short lifetime (about 12 ns). Due to the high quantum yield of intersystem crossing (Φ_{ISC} = 0.67), $^1RF^*$ can readily transform to triplet state ($^3RF^*$). By reason of the long lifetime (42 μs)[1], high excited energy (194.9 kJ·mol^{-1}) and high redox potential (1.7 V vs. NHE)[2] of $^3RF^*$, RF possesses quite complicated photochemical and photobiological properties. According to the previous studies, two mechanisms of type I and type II have been proposed to explain the photooxidation process of biomolecules caused by RF excited states. Type I mechanism involves direct reaction between $^3RF^*$ and a substrate via hydrogen atom or electron transfer. In type II mechanism, $^3RF^*$ can react with an oxygen molecule forming singlet oxygen ($^1O_2$) or superoxide anion radical through energy or electron transfer which can consequently damage the substrate[3,4]. The photooxidation of biomolecules causes the death of cell or cell apoptosis, resulting in age acceleration[5]. Previously, the photodynamic actions of RF on DNA have been extensively studied. Results have demonstrated that most RF damage DNA via electron transfer and the mechanism is different from porphyrin ($^1O_2$-mediated process)[6,7]. Research of protein photooxidation damage induced by RF is much less than that of DNA. In fact, photosensitizer can react with protein through complex photochemical reactions, giving rise to the change of protein, including structural and conformational modification, fragmentation[8], aggregation or cross-linking[1]. Flavin-sensitized photoprocesses in the eye lens have been mentioned as one of the causes of protein aggregation during aging and cataractogenesis[9]. Recently, the study

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of RF oxidative damage of a single amino acid or peptide has been carried out with many meaningful results obtained. However, the mechanism of RF photooxidative damage of protein is not completely understood due to the complexity of species and structure of protein. Actually, protein comprises approximately 68% of dry weight of cells and tissues, which leads to the probability that the photooxidation damage of protein is much more than that of DNA. Therefore, protein is potentially the major target for photooxidation damage.

As a potential drug in photodynamic therapy (PDT), RF was suggested to sensitize the killing of tumor cells and intra- and extra-cellular HIV[10,11]. Although a large amount of studies have been conducted on tumor destruction in PDT, the photochemical mechanisms are not well understood, especially on the photosensitized damage of protein. RF is an important vitamin and endogenous photosensitizer, whose study on photochemical reaction with protein is important and essential for elucidating its photodynamic mechanism.

In this study, lysozyme (Lyso) was selected as the model to observe the photooxidation damage of protein caused by RF using steady irradiation and SDS-PAGE, and the photodynamic mechanism was determined. The results will be helpful in understanding the mechanisms of photodynamic actions of RF on protein, providing useful theoretical evidence for PDT. We also have investigated the protective effect of different antioxidants on protein. Recently, the reported studies on the fast reaction dynamics have shown that antioxidants can not only scavenge deleterious radicals through competing reaction, but also repair the damaged biomolecules caused by radicals[12].

1 Materials and methods

1.1 Materials

Riboflavin (RF), melatonin (ML), epicatechin (EC), ascorbic acid (Vc), and chlorogenic acid (CA) were purchased from Sigma. Lyso (molecular weight is 14.6 kDa) was obtained from Fluka. All solutions were prepared freshly with pure water provided by Millipore purification system.

1.2 Instrumentation and experimental conditions

The experiments were carried out using 500 W xenon lamp as the light source of irradiation. Passing through a cut-off filter (bandwidth: 315—375 nm), the light was focused on quartz cuvettes and produced an irradiance of 34.3 $mW \cdot cm^{-2}$. The irradiated solutions were analyzed by SDS-PAGE (Bio-Rad Mini-PROTEIN® 3 Cell). Gels were then stained with Coomassie Brilliant Blue G-250 solution and destained in a solution containing methanol and acetic acid. The gels were subsequently scanned with Quantity one scanner (Bio-Rad). All absorbance measurements were performed in a UV-visible spectrophotometer (U-3010) with 300 $nm \cdot min^{-1}$ scan speed and 0.50 nm sampling interval.

2 Results

2.1 Wavelength of irradiation

The absorption spectra of RF and Lyso aqueous solution are shown in Figure 1. RF has two absorption peaks in UVA and visible light region principally due to π-π^* absorption[2]. Lyso has almost no absorption in the light with $\lambda > 315$ nm, therefore we chose the cut-off filter with 345 nm central wavelength and 60 nm bandwidth.

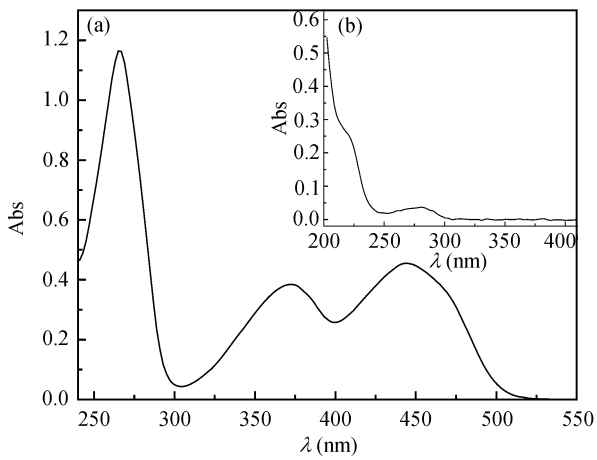

Figure 1 UV-visible absorption spectra. (a) 4×10^{-5} $mol \cdot L^{-1}$ RF aqueous solution; (b) 1×10^{-6} $mol \cdot L^{-1}$ Lyso aqueous solution.

2.2 Effect of ambience

The solutions of Lyso (5×10^{-4} $mol \cdot L^{-1}$) and the mixed solutions of RF (1×10^{-4} $mol \cdot L^{-1}$) and Lyso (5×10^{-4} $mol \cdot L^{-1}$) were irradiated for 30 min and continuously bubbled with nitrogen (N_2), air and oxygen (O_2), respectively. Irradiated solutions were analyzed by SDS-PAGE subsequently (Figure 2). 28—29 kDa products band was observed under three different ambiences, which was considered to be the dimer of Lyso for the molecular weight is twice of Lyso (14.6 kDa). Under the same at-

mosphere quantity of dimer increased obviously in the presence of RF. This demonstrated that RF can induce photodamage of Lyso. The damage of Lyso is greater in the irradiated solution bubbled with oxygen than with air, and even the distinct third band at 38—39 kDa can be observed under an oxygen atmosphere. In contrast, Lyso was lightly damaged when bubbled with N_2, which indicated that RF induced Lyso damage directly via type I photooxidation reaction. The damage of Lyso was enhanced exceedingly with the increasing of oxygen concentration (O_2 > air), which can be explained by photodynamic mechanisms that involved both type I and type II processes. Lyso damage was obviously greater in solutions bubbled with O_2 than with N_2. Indications showed that the damage of Lyso was the result of cooperation between type I and type II photodynamic mechanisms, and was dominated by the type II process. In addition, the observation of the third band at 38—39 kDa suggested cleavage reaction occurred besides cross-linking. The formed fragment can further react with dimer or other fragments to produce the third band.

2.3 Effect of concentration of RF

Figure 3 illustrates the gel electrophoresis pattern for the mixed solutions containing 5×10^{-4} mol·L^{-1} Lyso and RF of different concentrations irradiated for 30 min and bubbled with air continuously. It is important to note that the intensity of the dimer at 28—29 kDa increased gradually along with the increasing concentration of RF (from 5×10^{-7} to 7×10^{-5} mol·L^{-1}). The third band at 38—

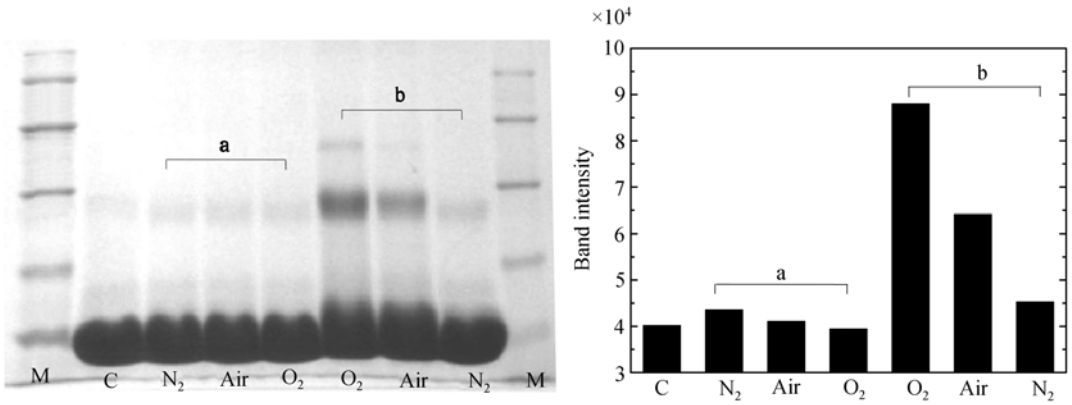

Figure 2 Gel electrophoresis pattern represents the effect of different ambiences on the photodamage of Lyso caused by RF. Irradiated for 30 min; a, 5×10^{-4} mol·L^{-1} Lyso bubbled with N_2, air and O_2, respectively; b, the solution containing RF (1×10^{-4} mol·L^{-1}) and Lyso (5×10^{-4} mol·L^{-1}) bubbled with N_2, air and O_2, respectively. C, control group; M, molecular weight marker (increase from 14.4, 20.1, 31, 43 to 66.2 kDa). The right figure is the intensity of bands at 28—29 kDa. The data represent the average of three different experiments.

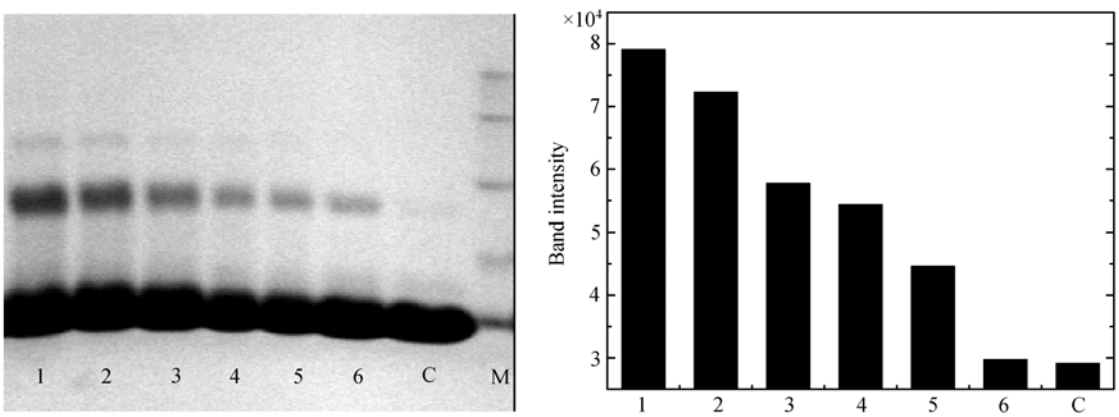

Figure 3 Gel electrophoresis pattern presents the effect of concentration of RF on the photodamage of Lyso. The mixed solutions which contained 5×10^{-4} mol·L^{-1} Lyso and different RF were irradiated with air bubbling for 30 min. 1, 7×10^{-5} mol·L^{-1} RF; 2, 4×10^{-5} mol·L^{-1} RF; 3, 1×10^{-5} mol·L^{-1} RF; 4, 2.5×10^{-6} mol·L^{-1} RF; 5, 5×10^{-7} mol·L^{-1} RF; 6, 0; C, control group; M, molecular weight marker (increase from 14.4, 20.1, 31, 43 to 66.2 kDa). The right figure is the intensity of bands at 28—29 kDa. The data represent the average of three different experiments.

39 kDa appeared when RF concentration was no less than 1×10^{-5} mol·L^{-1} (the molar ratio of Lyso and RF is 50), and the band intensity also increased with the increasing concentration of RF. Indications showed Lyso damage was strengthened at relatively high RF concentration, which can be explained by more excited RF enhanced photooxidation damage of Lyso via type I and type II processes.

2.4 Effect of time of irradiation

Figure 4 illustrates the gel electrophoresis pattern of the solutions containing 5×10^{-4} mol·L^{-1} Lyso and 5×10^{-5} mol·L^{-1} RF which were irradiated for different time and bubbled with air. The second band at 28—29 kDa was detectable after 10 min irradiation, and the third band at 38—39 kDa appeared when irradiation prolonged up to 30 min, and the forth band around 52 kDa was detected after 1 h irradiation. The results showed that the intensities of the new bands increased along time delay of irradiation. The effect of time of irradiation on Lyso damage was similar to that of RF concentration mentioned above. The longer irradiating time generated more excited states of RF which damaged Lyso via electron transfer and excited energy transfer.

2.5 Effect of antioxidants

The mixed solutions of Lyso (5×10^{-4} mol·L^{-1}) and RF (7×10^{-5} mol·L^{-1}) were irradiated for 30 min and bubbled with air in the presence of 5×10^{-4} mol·L^{-1} antioxidant of ML, EC, CA, and Vc, respectively. The gel electrophoresis pattern (Figure 5) was obtained after the samples were treated with the methods mentioned in sec. 1. It is noteworthy that the intensity of the 28—29 kDa band decreased in the presence of the antioxidant, and the third band at 38—39 kDa was not observed. The results indicated that antioxidants could effectively reduce Lyso damage. We can conclude that different efficiencies of the four antioxidants, CA>ML>Vc>EC, exist.

The rate constants of the reaction between $^3RF^*$ and antioxidants were listed in Table 1. Referring to the results in Table 1, we concluded that an antioxidant can not only interact with $^3RF^*$ but also react with $^1O_2$ under an aerobic atmosphere, and both reactions can protect Lyso from photooxidation damage. In addition, the possibility exists in repairing the damaged Lyso through a reaction with a Lyso radical.

The rate constant of the reaction between $^3RF^*$ and Lyso is 3.7×10^8 L·mol^{-1}·s^{-1}. Compared with quenching rate constants of $^3RF^*$ by antioxidants (listed in Table 1), results implied that the $^3RF^*$ can react with both antioxidants and Lyso within an anaerobic atmosphere, and these are two competing reactions. We calculated the proportion (Φ) of reaction of each antioxidant in the two competing reactions. The result showed Φ was more than 0.97 for all the four antioxidants. Results suggested that the protection of Lyso by antioxidants was mainly attributed to the direct reactions of $^3RF^*$ with antioxidants in anaerobic conditions.

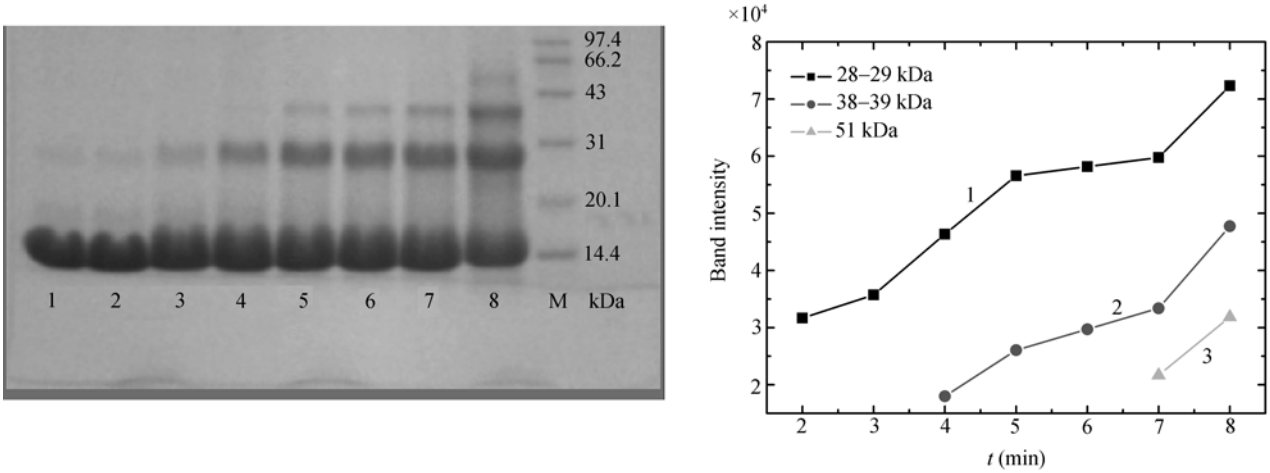

Figure 4 Gel electrophoresis pattern presents the effect of time of irradiation on the photodamage of Lyso. The solutions contained 5×10^{-4} mol·L^{-1} Lyso and 5×10^{-5} mol·L^{-1} RF which were irradiated for different time and bubbled with air. 1, Control group; 2, 0 min; 3, 10 min; 4, 20 min; 5, 30 min; 6, 40 min; 7, 50 min; 8, 60 min; M, molecular weight marker. The right figure illustrates the intensity of the new bands. 1, 28—29 kDa; 2, 38—39 kDa; 3, 51 kDa. The data represent the average of three different experiments.

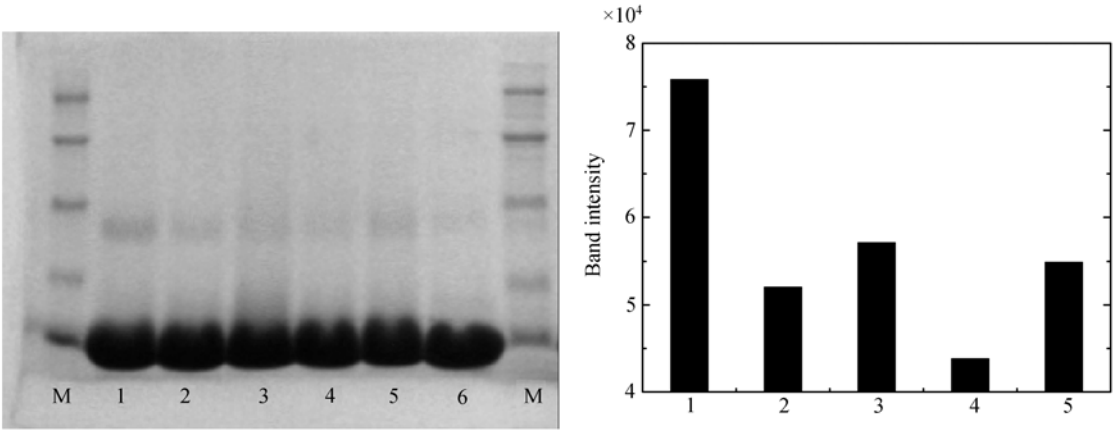

Figure 5 Gel electrophoresis pattern shows the effect of antioxidant on the photodamage of Lyso by RF. The mixed solutions of 5×10^{-4} mol·L^{-1} Lyso and 7×10^{-5} mol·L^{-1} RF with 5×10^{-4} mol·L^{-1} antioxidant were irradiated for 30 min and bubbled with air. 1, System without antioxidant; 2, system with antioxidant ML; 3, EC; 4, CA; 5, Vc; 6, control group; M, molecular weight marker (increase from 14.4, 20.1, 31, 43 to 66.2 kDa). The right figure is the intensity of band at 28—29 kDa. The data represent the average of three different experiments.

Table 1 Quenching rate constant of $^3RF^*$ and $^1O_2$ by antioxidant $k(^3RF^*)$ and $k(^1O_2)$, respectively, oxidation potential of antioxidant E_{ox}, free energy change ΔG for the reaction of $^3RF^*$ and antioxidant

Antioxidant	$k(^3RF^*)$ (10^9 L·mol^{-1}·s^{-1})	$k(^1O_2)$ (10^9 L·mol^{-1}·s^{-1})	E_{ox} (V)	$\Delta G^{a)}$ (kJ·mol^{-1})
ML	1.4[13]			
CA	2.1	3.4[14]	0.749 vs. NHE[14]	–94.5
EC	2.1	0.0132[15]	0.909 vs. NHE[15]	–79.0
Vc	1.6	0.8[16]	0.479 vs. NHE[17]	–120.5
Trp	1.8[18]		1.01 vs. NHE[19,20]	–68.5
Tyr	1.5[18]		0.93 vs. NHE[19,20]	–76.2

a) ΔG was calculated according to the Rehm-Weller equation.

3 Discussion

Lyso consists of 129 amino acids forming a single polypeptide chain, and the structure is quite stable. Three tyrosine residues are located inside and not readily exposed to the solvent, which is important in the structural stability of the Lyso[21]. Four of the six tryptophan residues are on the surface of the enzyme, while the other two are located in the active site of the enzyme and participate in catalytic process.

The results suggested that RF can induce photooxidation damage of Lyso under nitrogen ambience. The tryptophan residues in the native enzyme showed different exposure degree to the solvent[22]. The most exposed tryptophan residue is one of the major targets for photooxidation, which shows high reactive activity to $^3RF^*$[2,4]. The electron transfer reaction between $^3RF^*$ and tryptophan residue generated the Lyso radical, giving rise to bonds between two tryptophan residues, which can basically be responsible for protein cross-linking[4]. Therefore, in a N_2 saturated solution, RF damaged Lyso primarily due to the direct reaction between $^3RF^*$ and tryptophan residues in the enzyme (type I mechanism).

The quantum yields of $^1O_2$ and superoxide anion radical are 0.48 and 0.009 respectively in RF solution under air ambience[23]. The energy transfer reaction from $^3RF^*$ to ground state oxygen generating $^1O_2$ is dominant. The rate constant for chemical reactions of $^1O_2$ with different amino acids in side-chains vary dramatically, resulting in selective damage to particular residues. For example the rate constants of the reaction of tryptophan residues with $^1O_2$ is 3×10^7 L·mol^{-1}·s^{-1}[24]. Different exposure degree to the solvent of tryptophan residues in the native enzyme constitute the main targets for oxidative damage by $^3RF^*$ and $^1O_2$. In the presence of O_2, the generation of Lyso radical was attributed to the reaction between $^3RF^*$ (or/and $^1O_2$) and specific tryptophan residue. The bonds between two tryptophan residues are

mainly responsible for protein cross-linking. Increases in oxygen concentration, radiate time, and RF concentration can in turn increase the radicals' concentration, which accelerates the Lyso cross-linking. The investigation shows the Lyso damage under oxygen ambience is distinctly greater than in anaerobic conditions. Studies have suggested the damage of Lyso was mainly due to the cooperation of type II mechanism and type I mechanism. This mechanism is different from the process of RF damage to DNA through electron transfer (type I mechanism)[6].

Furthermore, the participation of other types of amino acids cannot be discarded. For example, the reaction of long-distance intramolecular electron transfer from tyrosine residues to tryptophan radicals can lead to the regeneration of the tryptophan residues and to the radical form of tyrosine[1]. Considering the presence of the third band at 38—39 kDa (in Figures 2 and 3), we determined that RF induced Lyso cleavage as well as aggregation, and the fragmentation could bind with the dimer generating higher molecular weight aggregates[8,9,18]. Further investigation of the photodamaged site of Lyso caused by RF will be carried out in the future.

Antioxidants can protect Lyso against damage by RF photosensitization. According to the results of fast reaction dynamics, the four antioxidants (ML, EC, CA, and Vc) protect Lyso against oxidative damage mainly due to direct interaction with $^3RF^*$ under nitrogen ambience. The relative protective efficiency of the four antioxidants mentioned above was CA>ML>Vc>EC under aerobic conditions. These antioxidants can react directly with $^3RF^*$ and $^1O_2$ to generate relatively stable phenoxyl radicals. Moreover, antioxidants may interact with damaged Lyso via electron transfer, and consequently repair the substrate[12,25].

4 Conclusion

RF can cause damage to specific amino acids, e.g. tryptophan and tyrosine, generating the radical form of the enzyme, and giving rise to cross-linking between two Lyso molecules. The oxidative damage of Lyso is enhanced by increasing RF concentration and radiating time. The presence of antioxidants can reduce Lyso cross-linking to some extent, and effectively prevent the photooxidation damage to Lyso. Different efficiencies of the four antioxidants were observed. ML and CA were more effective than EC and Vc.

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