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# Detection of Free Radical Transfer in Lipoxygenase I-B-Catalyzed Linoleic Acid−Soybean Protein Interaction by Electron Spin Resonance Spectroscopy (ESR)

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Effects of lipoxygenase I-B (LOX)-catalyzed oxidation of linoleic acid on soybean proteins was evaluated by electron spin resonance (ESR) and fluorescence spectroscopy in different model systems in the presence or absence of antioxidants. A strong central singlet signal was detected by ESR spectroscopy and identified as the carbon radical (*g* value range 2.0041−2.0054). A downfield shoulder attributed to the sulfur radical (*g* value 2.019−2.028) was also observed. The changes in soybean proteins were accompanied by an increase in fluorescence, indicating the formation of cross-links. Natural antioxidants such as ascorbic acid and α-tocopherol as well as synthetic antioxidants butyl hydroxytoluene (BHT) inhibited the development of both the free radical signal and the fluorescence when added to soybean proteins prior to incubation with linoleic acid and lipoxygenase I−B; the central singlet signal attributed to the carbon radical was reduced by 35−65%. This paper clearly indicates direct free radical transfer from oxidizing linoleic acid catalyzed by LOX to soybean proteins.

**KEYWORDS: Soybean proteins; lipoxygenase; linoleic acid; free radicals; ESR; fluorescence**

## INTRODUCTION

Radicals involved in lipid oxidation are short-lived species and can be measured by electron spin resonance (ESR) spectroscopy, which is the only method available for direct detecting of free radicals in biological systems (*1*). Detection of free radicals in food systems by ESR spectroscopy is widely used in the fields of food irradiation (*2*, *3*), lipid oxidation (*4*, *5*), antioxidants, and food processing (*6*), and the characteristic *g* value provides enough information to distinguish the carbon-, nitrogen-, and sulfur-centered radical (*7*). Saeed and Howell (*8*) and Saeed et al. (*9*) used this technique to show the transfer of free radicals from fish oil to proteins (including Atlantic mackerel myosin as well as ovalbumin, lysozyme, and amino acids) by ESR spectroscopy. Using the combined techniques of on-line high-performance liquid chromatography/electron spin resonance and mass spectrometry (LC/ESR and LC/MS), Qian et al. (*4*, *5*) identified spin trapped lipid-derived carbon-centered radicals formed in linoleic acid peroxidation and the reactions of two ω-6 polyunsaturated fatty acids (linoleic and arachidonic acids) with soybean lipoxygenase in the presence of α-[4-pyridyl 1-oxide]-*N*-*t*-butyl nitrone. Polat and Korkmaz (*10*) used ESR spectroscopy to study kinetics of radiation-induced free radical and $Mn^{2+}$ signals in γ-irradiated ground soybeans at a temperature range of 130−400 K. Lee et al. (*11*, *12*) investigated free radicals in γ-irradiated soybean paste by ESR spectroscopy and compared it with those of soybean protein isolate and soybean oil to examine the influences of irradiation dose, moisture content, and heating after irradiation of the free radical concentration and species.

The oxidized lipids in lipid−protein systems are considered to induce polymerization and aggregation of proteins, causing undesirable changes in the nutritional and functional properties of the proteins (*13*). It has been demonstrated that linoleic acid oxidation products induced by lipoxygenase were very reactive substances that may react with soybean proteins and that the structural characteristics of soybean proteins were affected by the interactions in a pronounced manner (*14*, *15*).

Commercial soybean protein isolates are prepared from low denatured defatted soybean flours with substantially high lipoxygenase activity (*14*). At the same time, the residual lipids present in soybean flours are typically 1% and are easily oxidized by soybean lipoxygenase during the preparation of soybean proteins. Boatright and Hettiarachchy (*16*) found that the lipids associated with soybean protein isolates contributed to protein insolubility and protein oxidation. Adding antioxidants to soybean protein isolates during processing resulted in a marked increase in protein solubility (*17*). Obata et al. (*15*) found that degradation of sulfhydryl groups in soymilk was related to the lipid oxidative reaction of lipoxygenases during soybean grinding.

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In a previous paper (*14*), we studied soybean protein aggregation induced by lipoxygenase-catalyzed linoleic acid oxidation. Soybean proteins obtained from the model systems consisting of soybean proteins and different levels of linoleic acid and lipoxygenase showed increased turbidity, protein oxidation, and surface hydrophobicity but decreased sulfhydryl and disulfide contents. These observations led us to propose that lipoxygenase-catalyzed linoleic acid oxidation products may react with soybean proteins via free radical transferring and that the structural characteristics of soybean proteins were affected by the interactions.

In this paper, we present the effect of lipids such as linoleic acid catalyzed by soybean lipoxygenase I-B (LOX) and pure linoleic acid (LA) from Sigma Chemical Corporation on soybean proteins in the presence and absence of antioxidants. Fluorescence spectroscopy was compared to investigate protein cross-linking and aggregation. The purpose was to study free radical transfer from oxidizing linoleic acid catalyzed by LOX to soybean proteins that results in protein denaturation and the formation of aggregates in the model system.

## MATERIALS AND METHODS

**Materials.** Low denatured, defatted soybean flour was obtained locally. The protein and fat contents were 57.36 and 1.93%, respectively. Soybean lipoxygenase type I-B (70 800 units/mg), linoleic acid (assay ≥99.0% (GC)), ascorbic acid, and α-tocopherol were all obtained from Sigma Chemical Co. Antioxidants BHT (butyl hydroxytoluene) and quinine sulfate were obtained from China Aroma Chemical Co., Ltd. (Hangzhou, China). All other reagents and chemicals were of analytical grade.

**Preparation of Lipid-Reduced Soybean Proteins (LRSP).** To obtain lipid-reduced soybean flour, defatted soybean flour, which had been ground to pass 80 meshes, was extracted with a mixed solvent of hexane and ethanol at 20 °C for 1.0 h with a flour, hexane, and ethanol ratio of 1:2:4. The slurry was vacuum filtered, and the filter cake was washed with 95% ethanol at 20 °C for 1.0 h with a flour solvent ratio of 1:5. Then, the slurry was vacuum filtered, and the cake was vacuum dried at the same temperature. The dried material was ground to pass through 80 meshes and then vacuum heated at 90 °C for 30 min to reinforce lipoxygenase inactivation. The obtained lipid-reduced soybean flour was suspended in distilled water in a liquid solid ratio of 10:1 and adjusted to pH 7.0 with 1 N NaOH, and the mixture was stirred for 30 min and then centrifuged at 1300*g* for 15 min at room temperature. The supernatant was adjusted to pH 4.5 with 1 N HC1 and centrifuged at 1300*g* for 15 min. After washing with water, the protein precipitate was resuspended in water and neutralized to pH 7.0 with 1 N NaOH. The samples were freeze-dried and stored in a cool place.

**Preparation of Oxidized Linoleic Acid for ESR Measurements.** Linoleic acid (1 mL) was poured into silica cuvettes and exposed to air for 7 days at 35 °C. The level of oxidation was monitored by measuring the peroxide value according to the method of Egan et al. (*18*). The peroxide values after 7 days for oxidized linoleic acid were 1679 MEq $kg^{-1}$ lipid.

**Preparation of Samples for ESR Spectroscopy.** Most of the studies were conducted on a model system using lipoxygenase. The procedure used is described next.

A total of 1.5 g of linoleic acid was suspended in 10 mL of Tris-HCl buffer (50 mM, pH 9.0) and 1 mL of 5 mM NaOH, and two drops of Tween 20 were added to it. The linoleic acid suspension was shaken up and diluted to 0.107 M with Tris-HCl buffer (50 mM, pH 9.0) before use.

The soybean lipoxygenase (LOX type I-B) suspension (1.8 mg of protein/mL) was prepared in phosphate buffer solution (0.1 M, pH 9.0).

Lipid-reduced soybean proteins (LRSP) were suspended in distilled water to obtain 5% (wt %) protein suspension and adjusted to pH 9.0 with 1 N NaOH.

A model system consisting of 8.5 mL of linoleic acid suspension (0.107 M), 10 mL of soybean LOX type I-B suspension, and 100 mL of 5% (wt %) protein suspension was shaken, and water was incubated at 30 °C for 0.5, 1.0, 2.0, 4.0, and 6.0 h, respectively. After incubation, the flasks were taken out, quick-frozen in liquid nitrogen, and then freeze-dried for 24 h in a LGJ10-C model freeze-drier (Four-Ring Science Instrument Plant Beijing, Beijing, China). The freeze-dried samples were stored in a desiccator at 25 °C over $CaSO_4$ to avoid interference from dielectric energy absorption by water. Freeze-dried samples prepared with this method were good at stabilizing and trapping radicals that required examination. Control systems either consisting of 100 mL of 5% (wt %) protein suspension added to 10 mL of soybean LOX type I-B suspension or 8.5 mL of linoleic acid suspension (0.107 M) or only 100 mL of 5% (wt %) protein suspension in the absence of both linoleic acid and soybean LOX type I-B were prepared in the same manner. Samples, in which LRSP was first mixed with antioxidants (BHT, vitamin C and E, or the mixtures of vitamins C and E) and then incubated with soybean LOX type I-B and linoleic acid, were also prepared alongside the previous samples.

**ESR Measurements.** The freeze-dried samples were ground to pass through 80 meshes, and sample aliquots (20 mg) were transferred to (3.5 mm i.d.) silica tubes. A JES-FA200 ESR Spectrometer (JEOL Ltd., Tokyo, Japan) with 100 kHz modulation was used to conduct the ESR analyses at room temperature under the following conditions: microwave frequency 9.06 GHz (X-band frequency), power 1 mW, central field 323.5 mT, sweep width 10 mT, modulation 0.5 mT, sweep time 60 s, and receiver gain in the range of 200−2000. Manganese oxide was used as a reference marker to calculate the *g* value between 1.9804 and 2.0357. To observe whether oxidizing linoleic acid catalyzed by lipoxygenase gave the signals under the previous conditions, a model system consisting of 8.5 mL of linoleic acid suspension (0.107 M), 10 mL of soybean LOX type I-B suspension, and 100 mL of distilled water was being shaken, and water was incubated at 30 °C for 0.5, 1.0, 2.0, 4.0, and 6.0 h, respectively. After incubation, the flasks were taken out and cooled to 0 °C by an ice bath. The mixture was filtered through a cellulose acetate membrane with a pore size of 0.45 $\mu$m (Satorious Company, Germany). The filtrate of 1.0 mL was transferred to silica tubes, freeze-dried for 24 h in a LGJ10-C model freeze-drier (Four-Ring Science Instrument Plant Beijing, Beijing, China), and then measured by an ESR spectrometer using the same parameters. A total of 0.5 mL of linoleic acid or 0.5 mL of oxidized linoleic acid, in which linoleic acid was exposed to air for 7 days at 35 °C, was transferred to silica tubes and measured by an ESR spectrometer using the same parameters mentioned previously. First-derivative spectra were recorded, and the data were processed using Microcal Origin 6.0 (Microcal Software, Inc.). The relative radical concentration was measured by comparing the intensities of the ESR signals of the samples being studied under the same conditions.

**Fluorescence Spectroscopy.** Organic solvent-soluble fluorescent products from soybean protein samples for ESR spectroscopy were extracted and analyzed according to Fletcher et al. (*19*). A total of 0.2 g of freeze-dried samples prepared for ESR spectroscopy was homogenized (FA25 model homogenizer, Fluko Equipment Shanghai Co., Ltd., Shanghai, China) with 20 mL of chloroform−methanol (2:1, v/v) at 45 °C for approximately 1 min. An equal volume of water was added, and after thorough vortex mixing, the samples were centrifuged for 10 min at 3000*g* (4 °C). The chloroform-rich layer was pipetted into a small screw-capped pyrex tube for subsequent fluorescence measurements. Fluorescence intensities were obtained on a Hitachi 650−60 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with an excitation at 360 nm (slit width 4 nm) and an emission maximum at 435 nm (slit width 5 nm). The fluorescence intensity of l $\mu$g $mL^{-1}$ quinine sulfate in a 0.1 M $H_2SO_4$ solution was used as a standard for measuring the relative fluorescence intensities of the samples. All samples were measured in triplicate, and the means were reported.

## RESULTS AND DISCUSSION

ESR spectra of LA, oxidized LA, and LA + LOX are shown in **Figure 1**. Neither pure LA nor oxidized LA gave signals under the testing conditions. Linoleic acid peroxide produced

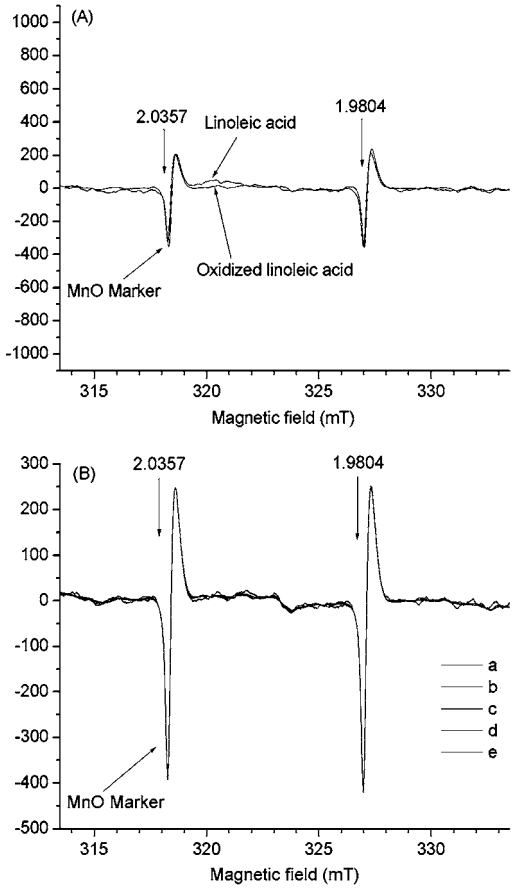


**Figure 1.** (**A**) ESR spectra of linoleic acid and oxidized linoleic acid. Oxidized linoleic acid was prepared by exposing linoleic acid (purchased from Sigma Chemical Co., assay ≥99.0% (GC)) to air for 7 days at 35 °C. The peroxide values after 7 days for oxidized linoleic acid were 1679 MEq $kg^{-1}$ lipid. (**B**) EPR spectra of linoleic acid peroxide produced in LOX-dependent linoleic acid peroxidation. The model system consisting of 8.5 mL of linoleic acid suspension (0.107 M), 10 mL of soybean LOX type I-B suspension, and 100 mL of distilled water was water incubated at 30 °C for (**a**) 0.5 h, (**b**) 1.0 h, (**c**) 2.0 h, (**d**) 4.0 h, and (**e**) 6.0 h, respectively.

in LOX-dependent linoleic acid peroxidation did not yet give signals under the same conditions. This means that the free radical signals observed in the following experiments existed only in the soybean proteins that were incubated with or without linoleic acid.

ESR spectra of soybean proteins incubated without added antioxidants are shown in **Figure 2**. When LRSP was incubated with LOX and LA for 2.0 h, the ESR spectra showed a sharp central free radical signal arising from the carbon-centered free radical (**Figure 2a**). The similarities of central singlet *g* values (range from 2.0041 to 2.0054), line widths, and line shapes in the signals observed here and elsewhere suggest carbon radicals either on the backbone of the −α-carbon or on the side chains. The intensity of the ESR signal increased with incubating time until LRSP was incubated with LOX and LA for 2.0 h. After 6 h incubation, the free radical signal of the sample decreased remarkably by 80% (**Figure 2e**). To understand further the free radical transfer from LA catalyzed by LOX to soybean proteins, the spectra of control samples under the same conditions were investigated. Control LRSP samples, in the presence of either LOX or LA, or in the absence of both LOX and LA, showed very minor radical peaks (**Figure 2b**−**d**).

Autoxidation of LA caused an ESR signal increase by 5% per hour as compared with the protein samples that contained

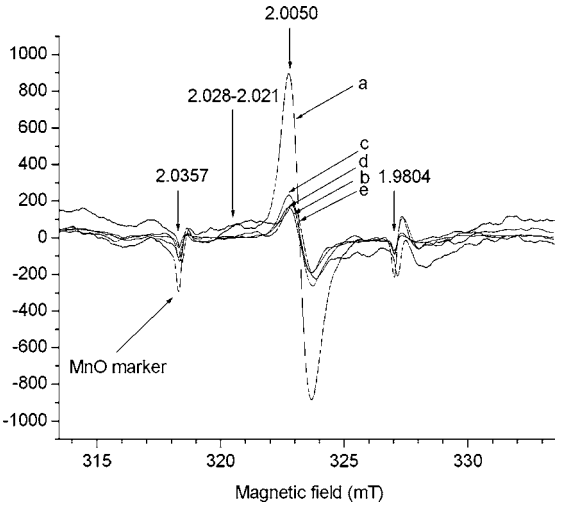


**Figure 2.** ESR spectra of soybean proteins incubated without added antioxidants. (**a**) Incubated with soybean LOX type I-B and linoleic acid for 2.0 h, (**b**) incubated without soybean LOX type I-B and linoleic acid for 2.0 h, (**c**) incubated with linoleic acid for 2.0 h, (**d**) incubated with soybean LOX type I-B for 2.0 h, and (**e**) incubated with soybean LOX type I-B and linoleic acid for 6.0 h.

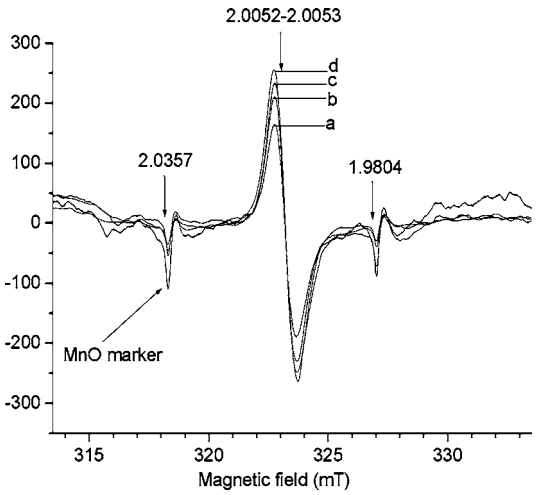


**Figure 3.** ESR spectra of soybean proteins incubated with or without linoleic acid. (**a**) Incubated without linoleic acid for 6.0 h, (**b**) incubated with linoleic acid for 2.0 h, (**c**) incubated with linoleic acid for 4.0 h, and (**d**) incubated with linoleic acid for 6.0 h.

neither LOX nor LA (**Figure 3a**−**d**). As a result, the intensity of the ESR signal of the LRSP + LA sample (2.0 h) (**Figure 2c**) was slightly higher than that of the LRSP or LRSP + LOX samples (**Figure 2b**,**d**). Since LA, oxidized LA, or LA + LOX on its own gave no signals in this study, the free radical signals observed were only produced when soybean proteins were present. In other words, the free radical detected here was transferred from LOX-catalyzed oxidizing LA to soybean proteins.

ESR spectra of soybean proteins incubated with added antioxidants are shown in **Figure 4**. Addition of BHT to LRSP prior to incubation with LA and LOX (**Figure 4b**) reduced the radical signal by 65% of the original size, while vitamin C (**Figure 4c**) and vitamin E (**Figure 4d**) reduced the peak by 34 and 33.5%, respectively. Using both vitamins C and E together decreased the level of intensity by 38% (**Figure 4e**). Although synthetic antioxidant BHT is suspected as a possible carcinogen (*20*), it inhibited the development of the free radical signal better

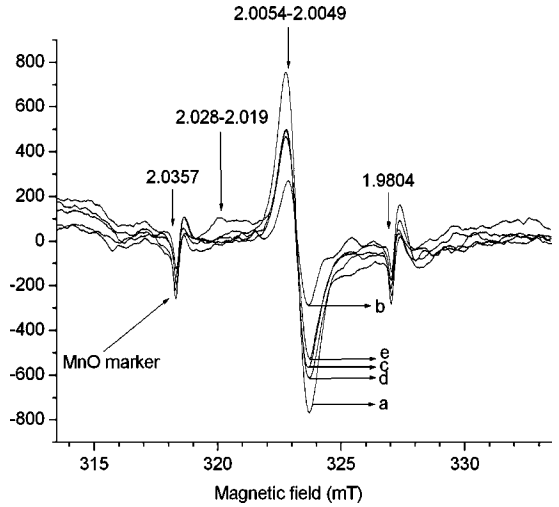


**Figure 4.** ESR spectra of soybean proteins incubated with added antioxidants. (**a**) Control incubated with soybean LOX type I-B and linoleic acid for 2.0 h without any added antioxidants, (**b**) mixed with BHT (200 ppm) and then incubated with soybean LOX type I-B and linoleic acid for 2.0 h, (**c**) mixed with vitamin C (500 ppm) and then incubated with soybean LOX type I-B and linoleic acid for 2.0 h, (**d**) mixed with vitamin E (500 ppm) and then incubated with soybean LOX type I-B and linoleic acid for 2.0 h, and (**e**) mixed with vitamins C and E (250 ppm:250 ppm) and then incubated with soybean LOX type I-B and linoleic acid for 2.0 h.

than natural antioxidants ascorbic acid and α-tocopherol when added to soybean proteins prior to incubation with LA and LOX.

In addition to the sharp central signal, there was an extra downfield signal observed on the spectra with *g* values ranging from 2.019 to 2.028 (**Figures 2a** and **4a**,**b**,**d**), suggesting the presence of the sulfur radical. It should be noticed that the intensity of the sulfur resonance obtained in our experiments was much lower than carbon radicals' signal. The sulfur radical had a short spin relaxation time as compared to other paramagnetic centers; thus, the microwave power used here could not make the sulfur radical saturate at this level. A progressively amplified sulfur resonance is observed by increasing the microwave power level from 1 to 50 mW, while the carbon radical signal decreased since they were easily saturated (data are not shown here). Partial sulfur oxidation may also contribute to the reduction of the signal as lipid peroxides could react with protein thiols to form sulfonic acid and other monomeric oxidation products that were not reduced by 2-mercaptoethanol (*21*).

Fluorescence spectra of the sample LRSP + LOX + LA reacting for different times is shown in **Figure 5**. The incubation of LRSP with LOX and LA at the beginning showed very little fluorescence (**Figure 5a**,**b**). However, after the first hour, the fluorescence increased sharply in the systems (**Figure 5c**,**d**,**e**). A reduction in the ESR signal occurred with the onset of fluorescence (**Figure 2a**,**e**), showing the formation of more stable bonds between either protein−lipid or protein−protein cross-links. As compared with LRSP + LOX + LA, the control samples, LRSP, LRSP + LA, and LRSP + LOX showed very little fluorescence during the whole incubation time (**Figure 6**).

Fluorescence spectroscopy has been widely used for studying lipid−protein interactions (*22*, *23*). Exposure of proteins to oxidized lipids is known to alter the amino acid sequence and protein conformation (*24*), leading to protein cross-linking. In particular, dityrosine formation from exposure to oxygen free radicals (*24*−*27*) exhibits fluorescence at an excitation of 320−360 nm and emission of 410−460 nm. Modifications to primary

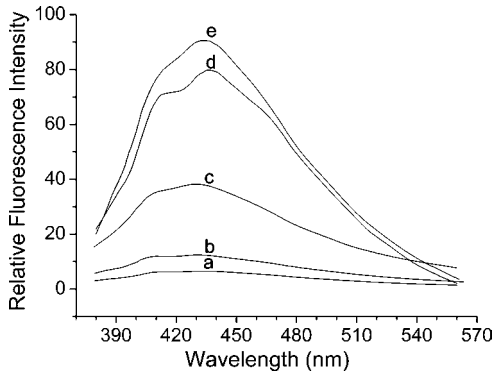


**Figure 5.** Fluorescence spectra of LRSP + LOX + LA mixtures after reaction extracted with chloroform–methanol (2:1, v/v) and fluorescence intensities relative to quinine sulfate in 0.1 M $H_2SO_4$ solution. Emission wavelength 435 nm and excitation wavelength 360 nm. The model system consisting of 8.5 mL of linoleic acid suspension (0.107 M), 10 mL of soybean LOX type I-B suspension, and 100 mL of 5% (wt %) protein suspension was water incubated at 30 °C for (**a**) 0.5 h, (**b**) 1.0 h, (**c**) 2.0 h, (**d**) 4.0 h, and (**e**) 6.0 h, respectively, and then freeze-dried.

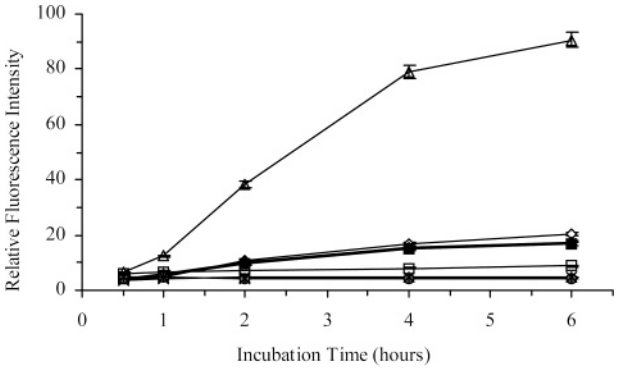


**Figure 6.** Fluorescence formation in LRSP and fluorescence intensities relative to quinine sulfate in 0.1 M $H_2SO_4$ solution. Emission wavelength 435 nm and excitation wavelength 360 nm. Bars represent means ± SD for $n = 3$. LRSP + LA + LOX + Vc + Ve (◇); LRSP + LA + LOX + Ve (■); LRSP + LA + LOX + Vc (▲); LRSP + LA + LOX + BHT (×); LRSP + LA + LOX (△); LRSP + LOX (◆); LRSP + LA (□); and LRSP (∗).

structure by oxygen radicals, such as bityrosine production and tryptophan loss, were also observed in all 16 widely different proteins (*26*). Liang (*23*) and Braddock and Dugan (*22*) observed an increase in fluorescence that is attributed to the formation of certain oxidized lipid−protein complexes, and some fluorescent compounds have been isolated from the oxidation reaction of linoleate and myosin in frozen Coho salmon (*22*). These compounds were shown to contain phosphorus and C═N functional groups. Here, a greater fluorescence was observed in LRSP + LA + LOX samples, indicating that bityrosine formation was at least one of the covalent forces that contributed to soybean protein aggregation induced by lipoxygenase-catalyzed linoleic acid oxidation.

The fluorescence produced by incubation of LRSP with LOX and LA in the presence of antioxidants was almost 80−90% less intense as compared with the samples incubated without BHT, vitamin C, or vitamin E (**Figure 6**). It showed that all antioxidants inhibited fluorescence development, while the effect of vitamin C, vitamin E, or mixtures of vitamins C and E on the inhibition was not as great as that shown for BHT. The formation of fluorescence in soybean proteins was evident in the systems incubated with LOX and LA. Both natural antioxidants such as vitamin C and vitamin E and synthetic antioxidant BHT inhibited the development of the fluorescence.

The mechanisms by which lipids and soybean proteins interact to produce aggregation during the preparation of soybean proteins are not completely understood. However, in this study, we have direct evidence by ESR spectroscopy that the major mechanism of LA oxidative damage to soybean proteins is due to the free radical transfer from LOX-catalyzed oxidizing LA to soybean proteins. Increasing fluorescence implied formation of cross-links by soybean proteins, which may affect the texture of soybean proteins during the preparation. Synthetic antioxidants (BHT), natural antioxidants (ascorbic acid and vitamin E), and combinations of vitamin E/ascorbic acid inhibited development of the free radical peak and fluorescence when added to soybean proteins prior to incubation with LA and LOX. These data show that soybean protein aggregation induced by LOX-catalyzed LA oxidation is mainly a free radical reaction. Oxidation of LA leads to the formation of organic free radicals that set off a destructive chain reaction in LA (*4*, *5*). Free radical transfer from oxidizing LA to soybean proteins results in the formation of large amounts of protein radicals. Protein free radicals may recombine to form the protein aggregates. However, the types of free radicals were not estimated from the ESR spectra except for carbon and sulfur radicals observed in the study. Further experiments should be focused on the measurement of free radical composition for the understanding of chemical reactions occurring in the model systems, as well as for the understanding of the mechanism of soybean protein aggregation induced by lipoxygenase-catalyzed linoleic acid.

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