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RADICAL CHROMATOGRAPHY OF SPIN ADDUCTS PRODUCED FROM γ-IRRADIATED LINOLEIC ACID IN NONAQUEOUS SOLUTION

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Spin adducts produced from γ-irradiated linoleic acid in an ethanol solution containing 2-methyl-2-nitrosopropane could be separated into nine fractions with high performance liquid chromatography. Distinct ESR spectra were observed from seven fractions and radical structures were identified for two major components.

A spin-trapping electron spin resonance (ESR) technique has been successfully applied to the detection and identification of short-lived free radicals formed in aqueous and nonaqueous solutions.[1] However, it is difficult to identify the trapped radicals in the presence of two or more spin adducts with closely similar ESR parameters. Recently, high performance liquid chromatography combined with ESR spectroscopy has made it possible to separate the coexisting spin adducts and to identify many radicals formed in γ-irradiated aqueous solutions of biomolecules such as amino acids and dipeptides.[2] So far, the application of this radical chromatography has been limited to γ-irradiated aqueous systems, though there are many fundamental biomolecules which are not sufficiently soluble in water and are soluble only in organic solvents.

In this study, the radical chromatography was extended so as to separate and identify the spin adducts of unstable radicals formed in γ-irradiated nonaqueous solutions of unsaturated carboxylic acids. Free radicals of linoleic acid are of biochemical interest associated with the lipid peroxidation *in vivo*.[3]

A spin trap reagent, 2-methyl-2-nitrosopropane (MNP) and di-*tert*-butylnitroxide (DTBN) were purchased from Aldrich Chemical Co. and Eastman Kodak Co., respectively, and used without further purification. Both 13-L-hydroxylinoleic acid (LA-OH) and 13-L-hydroperoxylinoleic acid (LA-OOH) were prepared and purified as described in previous papers.[4] Other chemicals were of commercial origin.

Linoleic acid and MNP were dissolved in ethanol and irradiated at room temperature. The concentration of linoleic acid and MNP was 100mM. Gamma-irradiation was carried out with ^{60}Co source to a total dose of 1×10^6 rad at a dose rate of 2.8×10^6 rad/hr.[5]

For the separation of the spin adducts, high performance liquid chromatography was carried out using an HLC-802 UR unit made by Toyo Soda Manufacturing Co.[4] Twenty μl of a sample solution was injected for chromatography.

A chromatogram obtained from γ-irradiated linoleic acid in an ethanol solution containing MNP is shown in Fig. 1. The effluent was divided into nine frac-

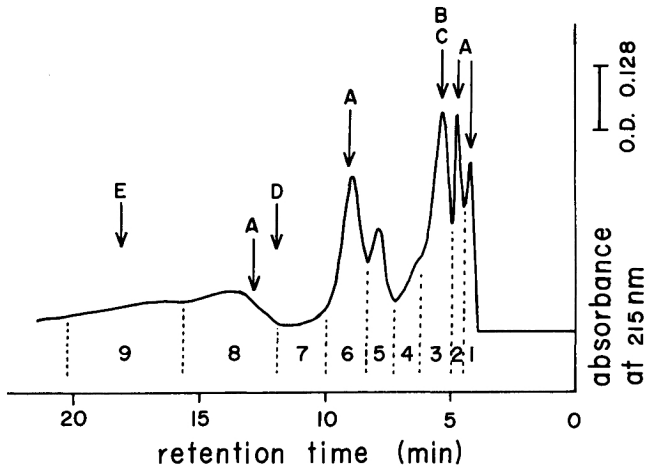

Fig. 1: Chromatogram obtained from γ-irradiated linoleic acid in an ethanol solution containing MNP. The effluent was divided into nine fractions (1-9). Arrows (A-E) show the retention places of following compounds; A: γ-irradiated MNP, B: DTBN, C: linoleic acid, D: LA-OH, and E: LA-OOH. Chromatographic conditions: column, porous polymer gel (TSK-Gel LS-141) column (4 x 600 mm) of Toyo Soda; eluent, n-hexane/ethanol (90%/10%); flow rate, 1.28 ml/min; pressure, 20 kg/cm^2.

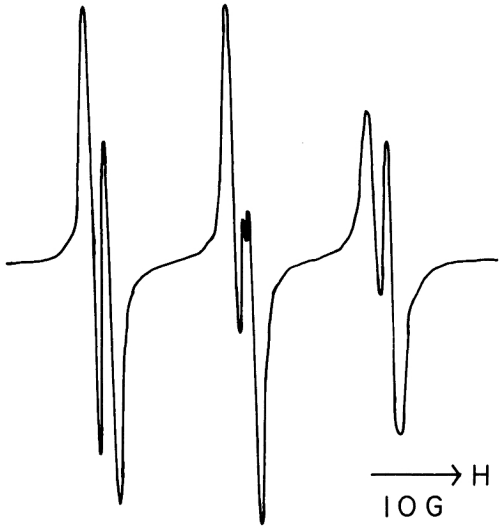

Fig. 2: ESR spectrum of γ-irradiated linoleic acid in the ethanol solution containing MNP. Conditions of ESR observation were as follows: gain amplitude, 2,000; modulation width, 0.25 G; response time, 0.3 s; microwave power, 3mW; and sweep rate of magnetic field, 100 G/ 8 min.

tions (1-9) according to the patterns of peaks or shoulders on the chromatogram. Each fraction was pooled and its solvent was evaporated by an aspirator for concentrating trace spin adducts. The concentrate was dissolved in 50% ethanol and ESR spectra of each fraction were recorded using an X-band ESR spectrometer(JEOL, Model FE-1X) operated at 100kHz modulation.

Chromatograms were also obtained from the following compounds using the same chromatographic conditions; γ-irradiated MNP in ethanol solution, DTBN, linoleic acid, LA-OH, and LA-OOH. The retention places of these compounds were designated by arrows in Fig. 1.

Among four retention places designated by arrows A, second one corresponding to a fraction 2 in Fig. 1 is due to both monomer and dimer MNP,[6] and the others are due to unidentified degradation products. Since a fraction 3 has exactly the same retention time as DTBN and linoleic acid, it is most likely that the fraction 3 does contain both DTBN and linoleic acid. A DTBN radical is expected to be formed by the self-trapping of tert-butyl radical from MNP.[1,6]

An ESR spectrum of γ-irradiated linoleic acid in the ethanol solution containing MNP is reproduced in Fig. 2. Apparently, it consists of, at least, two ESR patterns; one with a nitrogen hyperfine coupling constant (hfcc), a^N, of 15.9 G and a proton hfcc, a^H, of 1.5 G and the other with a^N of 16.7 G. However, it is rather difficult to assign a^N and a^H of both spin adducts definitely and to detect minor spin adducts of less intensity of ESR signal, because of the superposition

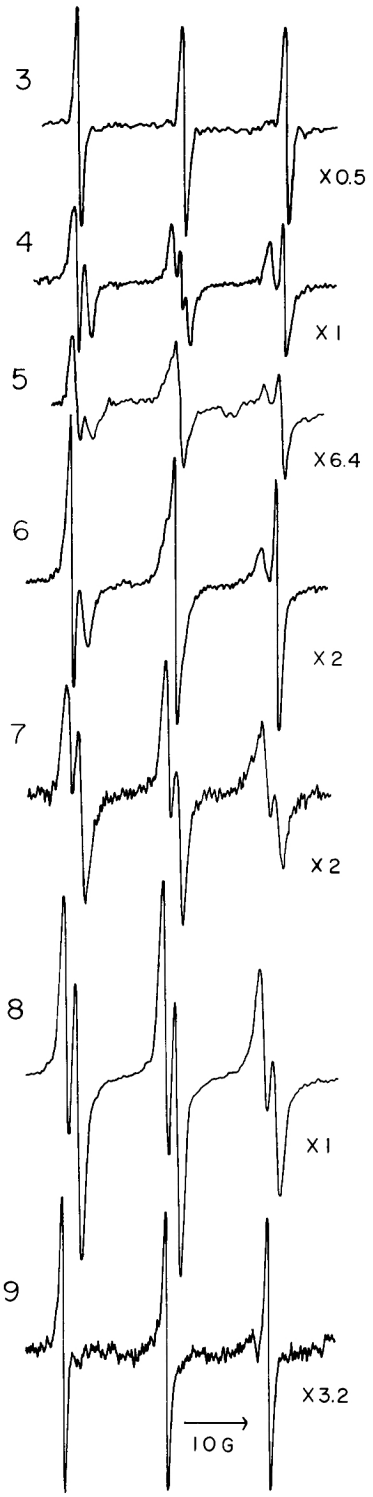

Fig. 3: ESR spectra of seven fractions (3-9) from the chromatogram in Fig. 1. Relative amplitude of ESR observation is drawn beside each spectrum.

of their ESR spectra.

In Fig. 3, ESR spectra of seven fractions (3-9) from the chromatogram in Fig. 1, are shown successively. In the ESR spectrum of a fraction 4, overlapping triplet and double-triplet patterns were observed. There were minor components with a double-triplet like pattern in fractions 5 and 6. Since fractions 1 and 2 did not give any discernible ESR spectra, these fractions do not contain spin adduct species. The fraction 2 could be ascribed to monomer and dimer MNP as described previously.

Observed hyperfine patterns and relative intensity of each ESR spectrum and assigned hfcc due to nitrogen and proton are tabulated in Table 1.

The nitrogen hfcc of DTBN was reported to be 17.12 G in water and 16.03 G in ethanol.[7] It depends mainly upon the polarity of solvents.[7] Therefore, it is estimated to be 16.58 G in 50% ethanol-50% water solvent, suggesting that the triplet ESR pattern with a^N of 16.7 G in a fraction 3 is due to DTBN. This assignment is consistent with the chromatographic view that the fraction 3 contains DTBN and linoleic acid.

A triplet component in the ESR spectrum of a fraction 4 may come from the tailing of a chromatographic peak of the fraction 3. Though ESR spectra of fractions 6 and 9 have the same triplet pattern with a^N of 16.7 G as of the fraction 3, they can't be ascribed to DTBN, because fractions 6 and 9 are clearly separated from fractions 3 and 4 as shown in the chromatogram in Fig. 1.

It is most probable that a fraction 8 contains the spin adduct from linoleic acid. During the course of this study, a chromatogram was obtained also from linoleic acid oxygenated by soybean lipoxygenase-1 in the presence of MNP. For comparison, the ESR spectra of each fraction were observed with the same range of the retention time as that in Fig. 1. Only fractions 3 and 8 gave almost identical ESR spectra, respectively, between γ-irradiation and enzymatic oxygenation systems of linoleic acid. These spectra from fractions 3 and 8 are two major components not only in the γ-irradiated system but also in the enzymatic one.

From the fraction 3 in the enzymatic system, an ESR spectrum of DTBN with a^N of 16.7 G was observed.

Table 1. ESR patterns, relative intensity, and hyperfine coupling constants assigned to ESR spectra of fractions 3-9.

Fraction No.	3	4	4	5	6	7	8	9
ESR pattern*	t	t	d-t	t	t	d-t	d-t	t
Relative intensity	4.7	0.7	1.5	0.2	1.6	1.3	3.5	1.0
a^N (G)	16.7	16.7	16.7	16.5	16.7	15.9	16.0	16.7
a^H (G)			1.4			1.4	1.7	

*) Letters t and d-t represent triplet and double-triplet, respectively.

From the fraction 8, a double-triplet pattern with a^N of 15.9 G and a^H of 1.5 G was observed. The major spin adduct from linoleic acid in the enzymatic system was once reported without chromatographic separation and the trapped radical species has been assumed to have radical center at position 13 and/or 9 of linoleic acid.[3,8] Therefore, the ESR spectrum of the fraction 8 is possibly ascribed to a spin adduct of an alkyl radical (-$\dot{C}$H-) at position 13 and/or 9 of linoleic acid.

At the present time, spin adducts in fractions 4, 5, 6, 7, and 9 could not be identified, although they were separated chromatographically and showed distinct ESR spectra.

In conclusion, high performance liquid chromatography of spin adducts has been extended successfully to a nonaqueous system and spin adducts from γ-irradiated linoleic acid in ethanol could be separated.

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