Journal of Chromatography, 487 (1989) 9–16
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4517

SEPARATION OF POLYUNSATURATED FATTY ACID RADICALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTRON SPIN RESONANCE AND ULTRAVIOLET DETECTION

RYOJIN SUGATA

Department of Biochemistry, Wakayama Medical College, 27 Kyubancho, Wakayama 640 (Japan)

HIDEO IWAHASHI

Department of Chemistry, Wakayama Medical College, 27 Kyubancho, Wakayama 640 (Japan)

and

TOSHIHIRO ISHII and RYO KIDO*

Department of Biochemistry, Wakayama Medical College, 27 Kyubancho, Wakayama 640 (Japan)

(First received July 28th, 1988; revised manuscript received September 30th, 1988)

SUMMARY

In the reaction of soybean lipoxygenase (EC 1.13.11.12) with polyunsaturated fatty acids such as linoleic, linolenic and arachidonic acids, some radical species were detected using the electron spin resonance (ESR) spin-trapping technique. The radical species derived from the three polyunsaturated fatty acids were not distinguishable because the ESR spectra of the spin adducts of nitrosobenzene with their three radical species showed no difference in their hyperfine splittings. To overcome this defect of the spin-trapping technique, these spin-adducts were separated by employing high-performance liquid chromatography (HPLC) combined with ESR spectroscopy. The spin adducts were eluted from a C_{18} reversed-phase column in the order linolenic acid, linoleic acid and arachidonic acid. The half-lives of the spin adducts separated by HPLC–ESR were determined as linoleic acid 600 min, linolenic acid 360 min and arachidonic acid 160 min. The use of an ultraviolet detector together with the HPLC–ESR technique resulted in a 500-fold increase in sensitivity in the detection of the radical species.

INTRODUCTION

Lipoxygenase is an enzyme that catalyses the conversion of polyunsaturated fatty acids containing a 1,4-*cis,cis*- pentadiene system to the lipid hydroperoxides [1–4]. This enzyme has been found in plants [5–7] and animals [8–15] and the role of this enzyme, e.g., as an initiating factor of the arachidonate cascade, has

0378-4347/89/$03.50

been investigated [16,17]. It is well known that some positional and stereochemical isomers of the hydroperoxides occur in the reactions of soybean lipoxygenase (EC 1.13.11.12) with linoleic acid, linolenic acid and arachidonic acid [18–23]. It has been suggested that the radical species are produced during the formation of the isomeric hydroperoxides [24]. Indeed, the radical species have been detected by using the electron spin resonance (ESR) spin-trapping technique [25–27], suggesting that a lone-pair electron is located at C-9 and/or C-13 in the linoleic acid radical [25,27]. Moreover using $^{17}O_2$, it was confirmed by Connor et al. [27] that carboncentre radical species are formed.

In order to separate and identify spin adducts, Makino and Hatano [28] proposed the combination of high-performance liquid chromatography (HPLC) with ESR spectroscopy (HPLC–ESR). By using this technique, retinoic acid radicals, the formation of which is catalysed by haemoglobin, were separated [29,30]. In this investigation, we used this technique to study the radical species formed in the reactions of soybean lipoxygenase with unsaturated fatty acids, because this technique allowed us to separate the radical species and to compare the three reactions of soybean lipoxygenase with linoleic, linolenic and arachidonic acids simultaneously.

EXPERIMENTAL

Materials

Linoleic, linolenic and arachidonic acids were purchased from Sigma (St. Louis, MO, U.S.A.). Lipoxygenase (type I) was obtained from Biozyme (Gwent, U.K.). Nitrosobenzene was a product of Nakarai Chemicals (Kyoto, Japan).

ESR spectroscopy

ESR spectra were recorded on a JEOL-FX2XG ESR spectrometer with the field set at 3365 ± 50 G, modulation frequency 100 kHz, modulation amplitude 1.0 G, recorder amplitude 5×1000, response 1.0 s, microwave power 20 mW and microwave frequency 9.435 GHz.

HPLC-ESR

HPLC–ESR chromatograms were achieved on a Jasco Trirotar-VI HPLC system with the above ESR spectrometer as a detector. A column (250 mm × 4.6 mm I.D.) packed with TSK-ODS gel (5 μm) was used with a flow-rate of 1.4 ml/min of methanol–10 m*M* sodium borate buffer (pH 8.0) (55:45, v/v). The column was kept at 20°C throughout. The magnetic field of the ESR spectrometer was fixed at the position ($g = 2.020$) indicated by the arrow in Fig. 1.

HPLC-UV

In order to improve the sensitivity of the detection of the radical species, a UV detector was combined with the HPLC–ESR system (HPLC–UV). The HPLC conditions using the HPLC–UV system were the same as those using the HPLC–ESR system. The wavelength of the UV detector was set at 300 nm.

Reaction mixture

A standard reaction mixture was prepared containing 0.2 *M* sodium borate buffer (pH 8.0), 0.8 m*M* polyunsaturated fatty acid, 6.6 m*M* nitrosobenzene and 1300 U/ml soybean lipoxygenase. Polyunsaturated fatty acid and nitrosobenzene were dissolved in ethanol and added to the reaction mixture. The final concentration of ethanol was 2.5% (v/v). The reactions were initiated by adding lipoxygenase and were carried out at 20°C for 30 s. Volumes of the reaction mixtures of 500 μl (HPLC–ESR) and 1 μl (HPLC–UV) were applied.

RESULTS

Detection of the radical species formed in the reactions of lipoxygenase with unsaturated fatty acids

A characteristic ESR spectrum with thirteen peaks was detected in the complete reaction mixture containing each unsaturated fatty acid, lipoxygenase and nitrosobenzene by using the ESR spin-trapping technique (Fig. 1a, d and e). Weak signals were observed in the absence of lipoxygenase (Fig. 1b) and no signals were observed in the absence of unsaturated fatty acid (data not shown) or nitrosobenzene (Fig. 1c).

The ESR spectra observed in the reactions of lipoxygenase with linoleic acid (Fig. 1a), linolenic acid (Fig. 1d) and arachidonic acid (Fig. 1e) showed similar hyperfine splittings.

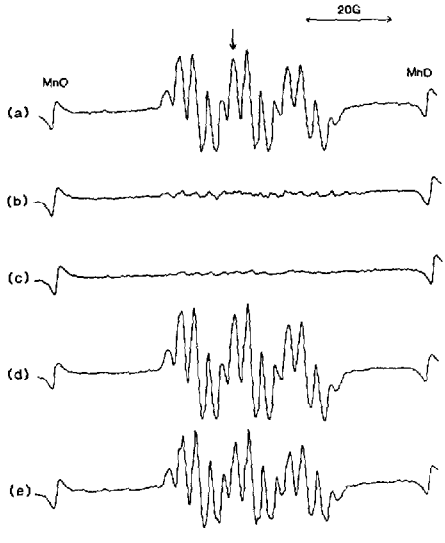

Fig. 1. ESR spectra of lipoxygenase–polyunsaturated fatty acid systems containing nitrosobenzene. (a) Spectrum of a system containing 0.8 m*M* linoleic acid, 6.6 m*M* nitrosobenzene and 1300 U/ml lipoxygenase in 0.2 *M* sodium borate buffer (pH 8.0); (b) spectrum of the system in (a) without lipoxygenase; (c) spectrum of the system in (a) without nitrosobenzene; (d) spectrum of the system in (a) with linolenic acid instead of linoleic acid; (e) spectrum of the system in (a) with arachidonic acid instead of linoleic acid. The arrow ($g=2.020$) in (a) indicates the position at which the magnetic field was fixed in HPLC–ESR spectrometry.

Separation of the radical species formed in the reaction mixtures by using the HPLC–ESR technique

In the HPLC–ESR elution profile of the reaction mixture of linoleic acid, two peaks with retention times (t_R) of 2.0 min and 14.6 min appeared in opposite directions with respect to the baseline (Fig. 2a). The peak with a retention time of 2.0 min was common to the elution profiles of the reaction mixtures of all the

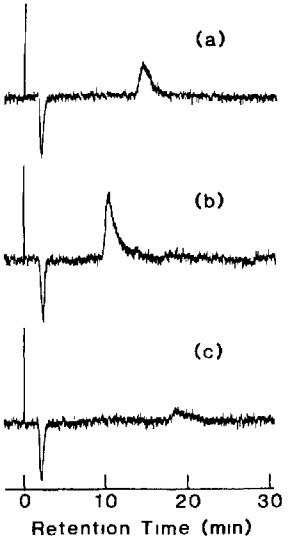

Fig. 2. HPLC–ESR traces of lipoxygenase–polyunsaturated fatty acid systems containing nitrosobenzene. Reaction conditions as in Fig. 1. (a) Linoleic acid; (b) linolenic acid; (c) arachidonic acid.

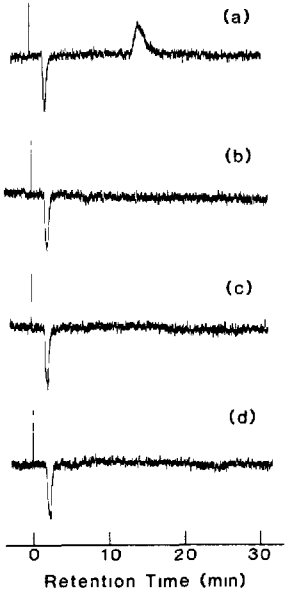

Fig. 3. HPLC–ESR traces of lipoxygenase–linoleic acid systems containing nitrosobenzene. Reaction conditions as in Fig. 1. (a) Complete reaction mixture; (b) as (a) but without lipoxygenase; (c) as (a) but without linoleic acid; (d) as (a) but without nitrosobenzene.

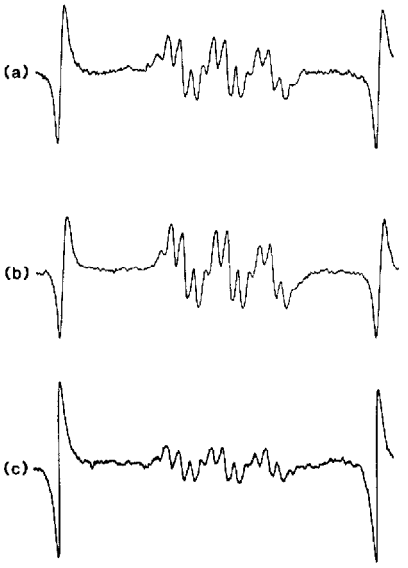

Fig. 4. ESR spectra of the separated radical species from the reaction mixtures of (a) linoleic acid, (b) linolenic acid and (c) arachidonic acid.

unsaturated fatty acids examined (Fig. 2). Prominent peaks other than this common peak were observed in the HPLC–ESR elution profiles of the reaction mixtures of linolenic acid at $t_R = 10.2$ min (Fig. 2b) and arachidonic acid at $t_R = 19.0$ min (Fig. 2c). Except for the common peak at $t_R = 2.0$ min, no peaks were observed in the absence of lipoxygenase (Fig. 3b), linoleic acid (Fig. 3c) or nitrosobenzene (Fig. 3d). The same was also observed with linolenic and arachidonic acids (data not shown). The ESR spectra of the respective HPLC–ESR peaks agreed completely with those of the reaction mixtures (Fig. 4).

Peaks other than the common peak at $t_R = 2.0$ min were not detected when 50% (v/v) methanol was used as the mobile phase and/or a column was kept at 40°C. The radical species formed in the reaction mixtures seem to be unstable.

Half-lives of the radical species separated by using the HPLC–ESR technique

The ESR intensity changes for the non-separated and separated radical species are shown in Fig. 5. The intensities of the non-separated radical species of the three unsaturated fatty acids decreased exponentially (Fig. 5a). The half-lives periods of the non-separated radical species are linoleic acid 30.2 min, linolenic acid 14.0 min and arachidonic acid 29.2 min. On the other hand, the intensity of the separated radical species decreased linearly (Fig. 5b). The half-lives of the separated radical species are linoleic acid 600 min, linolenic acid 360 min and arachidonic acid 160 min.

UV detection of the radical species

To detect the radical species, the HPLC–ESR technique required fairly large 500-μl portions of the reaction mixtures; no peak was detected when 1 μl was

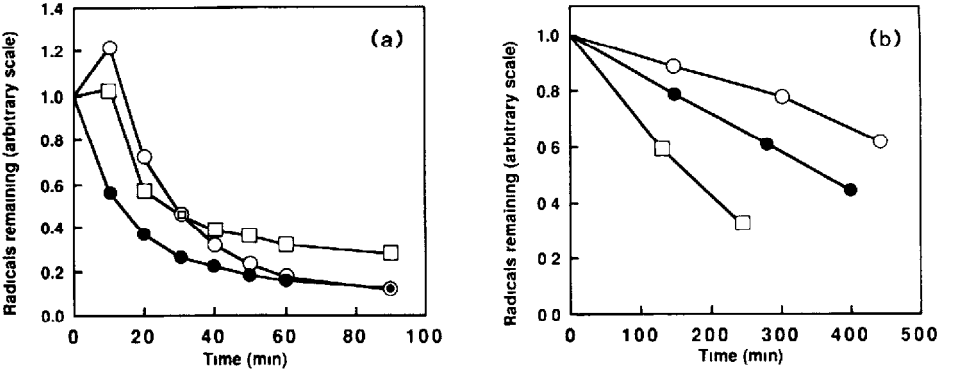

Fig. 5. ESR intensity changes of the non-separated and separated radical species. (a) Non-separated radical species; (b) separated radical species. (○) Linoleic acid; (●) linolenic acid; (□) arachidonic acid.

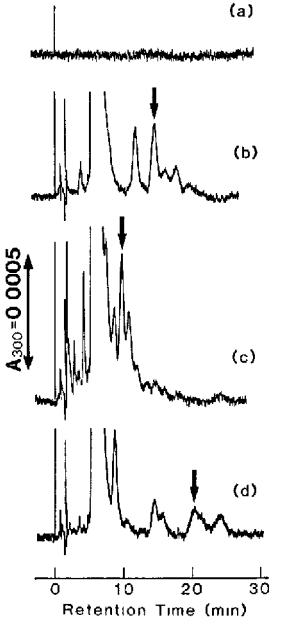

Fig. 6. HPLC-ESR and HPLC-UV traces of the lipoxygenase-polyunsaturated fatty acid systems containing nitrosobenzene. Reaction conditions as in Fig. 1 except for the volume injected (1 μl) (a) HPLC-ESR, linoleic acid; (b) HPLC-UV, linoleic acid; (c) HPLC-UV, linolenic acid; (d) HPLC-UV, arachidonic acid. The peaks corresponding to the respective radical species are indicated by arrows.

injected (Fig. 6a). On the other hand, when 1 μl of the reaction mixture of linoleic acid was subjected to the HPLC–UV method, the radical species were detected by the UV detector (Fig. 6b). The same results were observed with linolenic and arachidonic acids (Fig. 6c and d). The peaks of the radical species were assigned by comparing the HPLC–UV retention times with he HPLC–ESR data (Fig. 2a, b and c).

DISCUSSION

Some radical species that are formed during the peroxidation of linoleic, linolenic and arachidonic acids by lipoxygenase have been detected by using the ESR spin-trapping technique [26,27]. The ESR spectra were so similar in their hyperfine splittings that the radical species formed in the three reaction mixtures were not distinguishable.

To establish whether the radical species formed in the reactions are derived from the respective unsaturated fatty acids or not, the radical species were separated by using the HPLC–ESR technique. The peak with a retention time of 2.0 min, which was common in all the HPLC–ESR elution profiles (Fig. 2), may not come from the radical species, because injection of the reaction buffer also resulted in the appearance of this peak. This is probably due to the baseline change with elution of the reaction buffer. Prominent peaks other than the downward peak with a retention time of 2.0 min were observed at different retention times in the HPLC–ESR elution profiles of the three reaction mixtures. The prominent peaks seem to come from the reaction products in which bonds are formed between the nitrogen atom of nitrosobenzene and C-13 (for linoleic and linolenic acids) or C-15 (for arachidonic acid), because the ESR spectra of the peaks agree completely with those of the corresponding reaction mixtures (Fig. 4). This finding suggests that different radical species are formed in the three reaction mixtures. From the investigations using $^{17}O_2$ and under anaerobic conditions, Connor et al. [27] also concluded that fatty acid-derived radical were formed in the reaction of linoleic acid with lipoxygenase.

The HPLC–ESR technique allowed us to determine the half-lives of both separated and non-separated radical species. Separation of the radical species increased the half-lives. The longer half-lives of the separated radical species may be due to the absence of compounds that could react with the radical species or to a solvent change from water to 55% methanol. The separation of the radical species by the HPLC–ESR technique did not succeed under conditions such as a low methanol concentration, a low pH of the mobile phase and/or a high column temperature. Hence various factors seem to influence the half-lives of the radicals.

Detectors with high sensitivity are necessary to detect the radical species formed in biological systems containing very low concentrations of these species. In order to improve the sensitivity, we employed a UV detector with the HPLC–ESR technique. The sensitivity was improved 1500-fold compared with the HPLC–ESR analysis. In addition, the use of the HPLC–UV technique resulted in good separation of the peaks because of the small volume of sample injected. Small peaks with retention times 16.1 and 17.5 min can be seen behind the main peak ($t_R = 14.6$ min) in the HPLC–UV elution profile of the reaction mixture of lipoxygenase with linoleic acid (Fig. 6c). In the absence of lipoxygenase the small peaks were not observed. The results indicate that the small peaks are due to spin adducts of nitrosobenzene with some radical species. The small peaks may be due to radical species which are positional and stereochemical isomers [25,27]. The peaks observed in front of the main peak are probably due to the reagents or the reaction products of lipoxygenase with linoleic acid. Well separated peaks of the radical

species were also observed in the HPLC–UV analysis of reaction mixtures of linolenic and arachidonic acids.

In conclusion, HPLC–UV analysis is a powerful method of the separation of radical species with similar structures. Further, the structures of radical species might be determined using the HPLC–ESR technique in combination with other techniques such as mass and NMR spectrometry.

REFERENCES

1 G.A. Veldink, J.F.G. Vliegenthart and J. Boldingh, Prog. Chem. Fats Other Lipids, 15 (1977) 131.
2 K.H. Fischer and W. Grosch, Z. Lebensm.-Untersch.-Forsch., 165 (1977) 137.
3 T. Galliard and H.W.-S. Chan, in P.K. Stumpf (Editor), The Biochemistry of Plants, A Comprehensive Treaties, Vol. 4, Academic Press, New York, 1980, p. 131.
4 J.F.G. Vliegenthart and G.A. Veldink, in W.A. Pryor (Editor), Free Radicals in Biology, Vol. V, Academic Press, New York, 1982, p. 29.
5 A. Pinsky, S. Grossman and M. Trop, J. Food Sci., 36 (1971) 571.
6 H.W. Gardner, in M.G. Simic and M. Karel (Editors), Autoxidation in Food and Biological Systems, Plenum Press, New York, 1980, p. 447.
7 S. Ida, Y. Masaki and Y. Morita, Agric. Biol. Chem., 47 (1983) 637.
8 M. Hamberg and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 3400.
9 D.H. Nugteren, Biochim. Biophys. Acta, 380 (1975) 299.
10 P. Borgeat, M. Hamberg and B. Samuelsson, J. Biol. Chem., 251 (1976) 7816.
11 R.C. Murphy, S. Hammarström and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 4275.
12 M.I. Siegel, R.T. McConnell, N.A. Porter and P. Cuatrecasas, Proc. Natl. Acad. Sci. U.S.A., 77 (1980) 308.
13 O. Radmark, C. Malmsten and B. Samuelsson, Biochem. Biophys. Res. Commun., 96 (1980) 1679.
14 T. Yoshimoto, Y. Miyamoto, K. Ochi and S. Yamamoto, Biochim. Biophys. Acta, 713 (1982) 638.
15 R.W. Bryant, J.M. Bailey, T. Schewe and S.M. Rapoport, J. Biol. Chem., 257 (1982) 6050.
16 B. Samuelsson, Science, 220 (1983) 568.
17 B. Samuelsson, Angew. Chem. Int. Ed. Engl., 22 (1983) 805.
18 M. Hamberg and B. Samuelsson, J. Biol. Chem., 242 (1967) 5329.
19 G.A. Veldink, G.J. Garssen, J.F.G, Vliegenthart and J. Boldingh, Biochem. Biophys. Res. Commun., 47 (1972) 22.
20 M.O. Funk, R. Isaac and N.A. Porter, Lipids, 11 (1976) 113.
21 A. Yamamoto, Y. Fujii, K. Yasumoto and H. Mitsuda, Lipids, 15 (1980) 1.
22 E. Kaplan and K. Ansari, J. Chromatogr., 350 (1985) 435.
23 J.I. Teng and L.L. Smith, J. Chromatogr., 350 (1985) 445.
24 G.J. Garssen, J.F.G., Vliegenthart and J. Boldingh, Biochem. J., 122 (1971) 327.
25 J.J.M.C. De Groot, G.J. Garssen, J.F.G. Vliegenthart and J. Boldingh, Biochim. Biophys. Acta, 326 (1973) 279.
26 H. Aoshima, T. Kajiwara, A. Hatanaka and H. Hatano, J. Biochem., 82 (1977) 1559.
27 H.D. Connor, V. Fischer and R.P. Mason, Biochem. Biophys. Res. Commun., 141 (1986) 614.
28 K. Makino and H. Hatano, Chem. Lett., (1979) 119.
29 H. Iwahashi, A. Ikeda, Y. Negoro and R. Kido, Biochem. J., 236 (1986) 509.
30 H. Iwahashi, Y. Negoro, A. Ikeda and R. Kido, J. Chromatogr., 391 (1987) 199.