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CONVERSION OF 9-D- and 13-L-HYDROPEROXYLINOLEIC ACIDS BY SOYBEAN LIPOXYGENASE-1 UNDER ANAEROBIC CONDITIONS

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Summary

Soybean lipoxygenase-1 reacts with both 9-D- and 13-L-hydroperoxylinoleic acids under anaerobic conditions.

Approximately 40% of the hydroperoxide is converted into oxodienes, absorbing at 285 nm. Concomitantly, more polar compounds are formed, tentatively identified as being mainly epoxy-hydroxy-octadecenoic acids.

When oxygen is present, the reaction is strongly inhibited, until in a very slow reaction the oxygen has been depleted. This accounts for the occurrence of a lag period.

Introduction

The existence of a system that converts hydroperoxides in the living cell is most likely when hydroperoxides are produced in vivo. Otherwise decomposition of hydroperoxides could initiate chain reactions, which can cause serious damage to proteins, e.g. disruption of membranes and inactivation of enzymes [1—3]. For this reason, considerable attention has been paid to the conversion of hydroperoxides by enzymic or non-enzymic systems.

In various plant seeds, the enzyme lipoxygenase (EC 1.13.11.12), which in vitro oxidizes unsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system to hydroperoxides, occurs in relatively high concentrations.

A review has recently been published by Gardner considering the degradation of hydroperoxides [4]. In those cases where the involvement of lipoxygenase has been demonstrated, mostly unpurified enzyme preparations have been used. Lipohydroperoxidase activity of purified lipoxygenase-1, as reported so far, was found to be dependent on the presence of a suitable hydrogen donor, e.g. guaiacol under aerobic conditions (Grosch et al., [5] and Streckert et al. [6]), or linoleic acid under anaerobic conditions (Garssen et al. [7,8]). In

this paper, a lipohydroperoxidase activity of purified soybean lipoxygenase-1 is reported, which does not require the addition of an extra hydrogen donor.

Materials and Methods

Lipoxygenase-1 was isolated from soybeans according to Finazzi-Agrò et al. [9]. Spec. act.: 160 μmol O_2 $min^{-1} \cdot mg^{-1}$.

Linoleic acid (purity > 99%) was obtained from Lipid Supplies (St. Andrews University, St. Andrews, Scotland).

[U-^{14}C]linoleic acid (948 Ci/mol) was purchased from New England Nuclear (Boston, Mass., U.S.A.).

13-L- and 9-D-hydroperoxylinoleic acids were prepared by aerobic incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0 [7] or corn-germ lipoxygenase at pH 6.6 [10] respectively. The hydroperoxides were isolated by preparative thin-layer chromatography and stored in ethanol at −20°C. Under these conditions, they were stable for at least seven months. Both preparations were only for a few percent contaminated with isomeric hydroperoxides.

For the preparation of the [U-^{14}C]labelled hydroperoxides, [U-^{14}C]-linoleic acid (2.8 Ci/mol) was used.

A molar absorption coefficient of 25 000 $M^{-1} \cdot cm^{-1}$ at 234 nm was used for the hydroperoxylinoleic acids. Molar absorption coefficients at 285 nm of 30 000 $M^{-1} \cdot cm^{-1}$ and 22 000 $M^{-1} \cdot cm^{-1}$ were used for 13-oxotridecadienoic acid and 13-oxooctadecadienoic acid respectively [7].

Thin-layer chromatography was carried out on 0.25 mm or 0.50 mm (preparative) pre-coated plates (Silicagel 60F-254, 20 × 20 cm, E. Merck, A.G., Darmstadt, Germany).

Spectrophotometric measurements were carried out in a 1-cm pathlength cuvet provided with a rubber seal-cap. Prior to the incubation the cuvet was flushed with oxygen-free argon for 15 min. Preformed hydroperoxylinoleic acid in ethanol (volume <4 μl) was then added and the cuvet completely filled (4.25 ml) with deaerated 0.1 M sodium borate buffer (pH 9.0) via the seal-cap. The reaction was started by adding enzyme and the change in absorption at 285 or 234 nm was recorded with a Unicam SP1800 spectrophotometer. Spectra were recorded before and after reaction. All assays were performed at 25°C.

In some experiments, a dual-purpose cuvet of 1-cm light-path was used (as described by Garssen et al. [7]), which enabled the simultaneous recording of both the oxygen consumption and the change in absorption at a selected wavelength.

Incubations with [U-^{14}C]labelled hydroperoxides were carried out as follows: approximately 0.3 μmol of 13-L-[U-^{14}C]hydroperoxylinoleic acid or 9-D-[U-^{14}C]-hydroperoxylinoleic acid (2.8 Ci/mol) were dissolved in 4 ml 0.1 M sodium borate buffer (pH 9.0). The precise hydroperoxide concentration was determined spectrophotometrically by measuring the absorption at 234 nm. The incubation tube was closed with a seal-cap and the solution was deaerated by repeated evacuation and flushing with oxygen-free nitrogen gas. The reaction was started by the addition of soybean lipoxygenase-1 via the seal-cap. The incubations were carried out at 25°C. The reactions were terminated by acidification to pH 2, followed by extraction with diethylether. The washed extracts

were dried and concentrated. The residues were treated with diazomethane and subjected to thin-layer chromatography in the solvent system hexane-diethylether (60 : 40, v/v). After thin-layer chromatography, the distribution of the ^{14}C-label was determined by scanning the plates with a Berthold Dünnschichtscanner II, provided with an electronic integrator. To identify carbonyl compounds, in some experiments the plates were sprayed with a 0.4% (w/v) dinitrophenyl-hydrazine solution in 2 M HCl.

Results

The anaerobic conversion of 13-L- and 9-D-hydroperoxylinoleic acids by lipoxygenase-1 in the absence of linoleic acid proceeds much slower than the anaerobic conversion of 13-L-hydroperoxylinoleic acid in the presence of linoleic acid [7,8]. Reasonable reaction rates were obtained with 20—40 fold higher enzyme concentrations. Under these conditions a complete conversion of the hydroperoxides resulted, as was shown by thin-layer chromatography. Fig. 1 presents the ultraviolet absorption spectra before and after reaction. A decrease of 83% (13-L-) or 88% (9-D-) of the initial absorbance at 234 nm was observed *. A new absorption maximum appeared at 285 nm. 9-D-Hydroperoxylinoleic acid was found to be a much better substrate than the 13-L-isomer. A linear relation between the velocity of the reaction and the enzyme concentration was observed (Fig. 2). However, at very high concentrations of lipoxygenase, a deviation from linearity was found with the 9-D-isomer as substrate. No reaction was observed when the enzyme was first inactivated by heating at 85°C for 5 min in a 2% sodium dodecyl sulphate solution. The addition of sodium dodecyl sulphate was necessary to keep the protein in solution. Sodium dodecyl sulphate as such did not interfere with the reaction.

The anaerobic reaction of both hydroperoxides with lipoxygenase-1, showed a similar pH profile (optimum at pH 9) as the aerobic conversion of linoleic acid by this enzyme [11].

In all experiments a kinetic lag phase was observed, which corresponds to

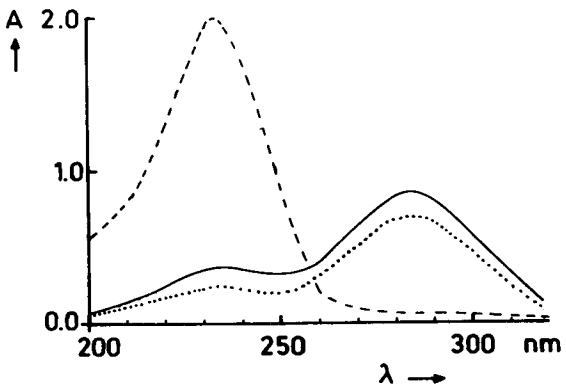

Fig. 1. Ultraviolet spectra before (------) and after the anaerobic conversion of 13-L-hydroperoxyoctadecadienoic acid (80 μM (———) and 9-D-hydroperoxyoctadecadienoic acid (80 μM) (. . . . .) by lipoxygenase-1 (1.0 μM).

* Reactions were complete after 120 min (13-L-) or 5 min (9-D-).

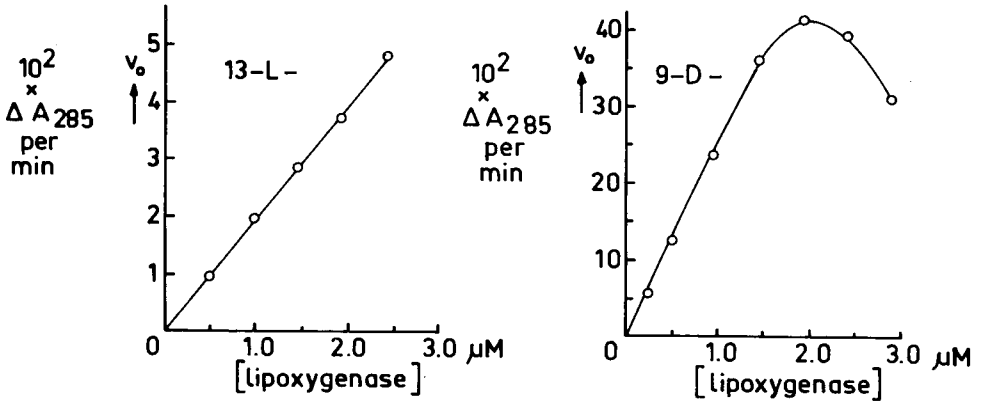

Fig. 2. Dependence of A 285 formation on the concentration of lipoxygenase-1. [13-L-hydroperoxyoctadecadienoic acid] : 80 μM, [9-D-hydroperoxyoctadecadienoic acid]: 80 μM.

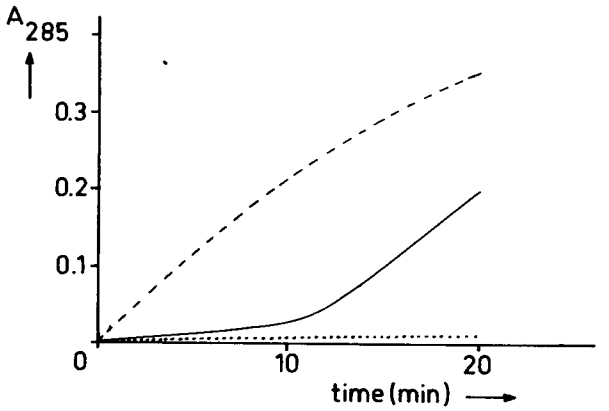

Fig. 3. Effect of oxygen on the length of the induction-period in the anaerobic conversion of 13-L-hydroperoxyoctadecadienoic acid (47 μM) by lipoxygenase-1 (1.5 μM). ------, 0 μM O_2 (obtained by addition of 5 μM linoleic acid); ———, 2 μM O_2; . . . . . , 20 μM O_2.

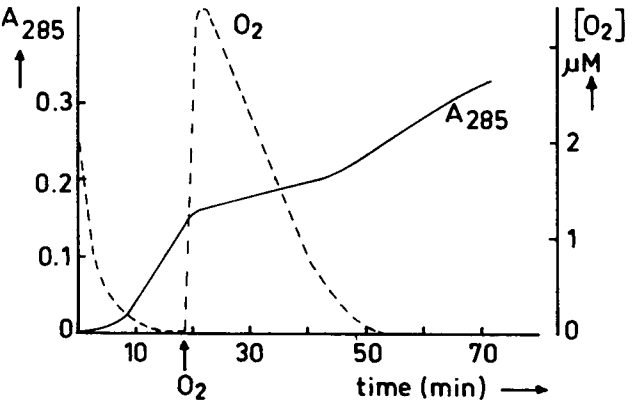

Fig. 4. Relationship between oxygen concentration and the rate of A 285 formation from 13-L-hydroperoxyoctadecadienoic acid (65 μM) by lipoxygenase-1 (1.0 μM). After 19 min, an oxygen pulse was given by the addition of air-saturated borate buffer.

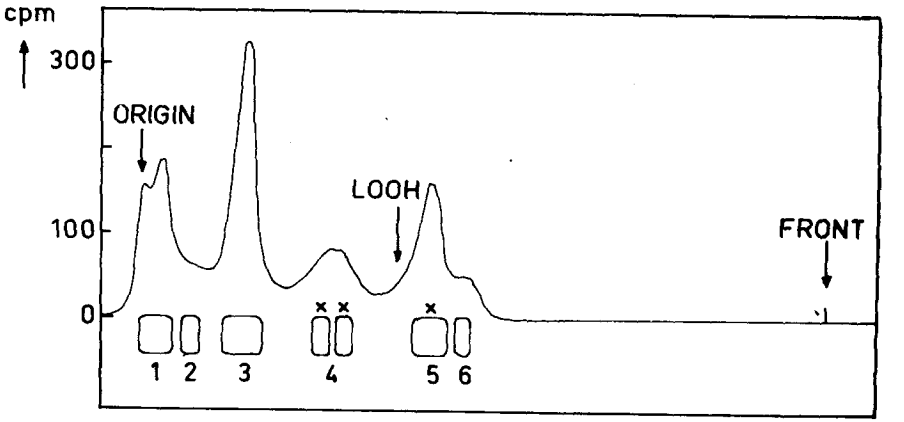

Fig. 5. Thin-layer radiochromatogram of the products formed in the anaerobic reaction between [U-^{14}C]-13-L-hydroperoxyoctadecadienoic acid (76 μM) and lipoxygenase-1 (2.0 μM). Reaction time: 80 min. Bands were stained by spraying with a phosphomolybdic acid solution. X: band coloured orange when sprayed with dinitrophenylhydrazine. LOOH: hydroperoxyoctadecadienoic acid.

Zone	R_F value	Mean percentage of total radioactivity	± S.D. [4]
1	origin	19.1	± 3.3
2	0.07	8.6	± 1.5
3	0.15	31.5	± 0.5
4	0.27/0.29	18.1	± 1.4
5	0.42	20.1	± 2.6
6	0.47	2.6	± 1.0

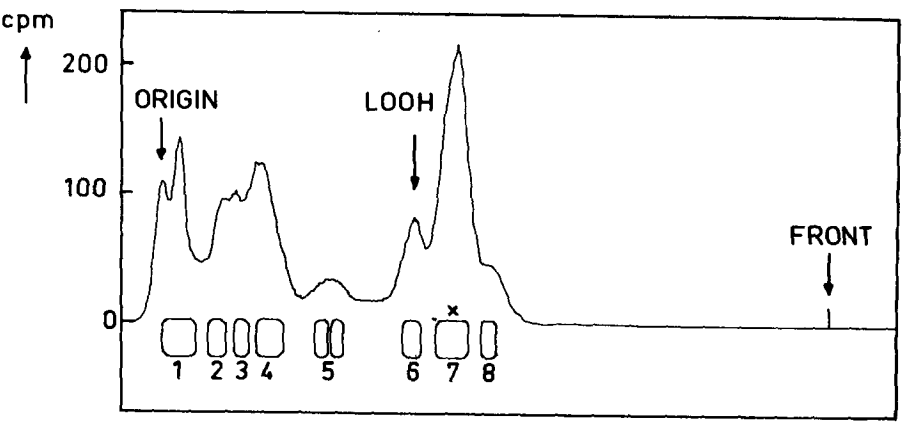

Fig. 6. Thin-layer radiochromatogram of the products formed in the anaerobic reaction between 9-D-[U-^{14}C]hydroperoxyoctadecadienoic acid (78 μM) and lipoxygenase-1 (0.5 μM). Reaction time: 20 min. Bands were stained by spraying with a phosphomolybdic acid solution. X: band coloured orange when sprayed with dinitrophenylhydrazine. LOOH: hydroperoxyoctadecadienoic acid.

Zone	*R_f value*	*Mean percentage of total radioactivity*	*[2 experiments]*
1	origin	18.8	
2 + 3	0.09/0.11	15.2	
4	0.14	20.2	
5	0.24/0.26	6.1	
6	0.37	8.5	
7	0.44	28.2	
8	0.49	3.0	

the time necessary to consume traces of oxygen (<2 μM) still present after deaeration. The addition of 5 μM linoleic acid eliminated the lag period completely, whereas addition of 20 μM oxygen caused a considerable extension (Fig. 3). During the lag time a slow reaction took place between oxygen, hydroperoxide and enzyme, which resulted in the complete removal of oxygen. Direct evidence for this new reaction was obtained by using the dual-purpose cuvet. Maximum reaction velocity was reached when the oxygen concentration dropped below 0.2 μM. In a typical experiment, visualized in Fig. 4, an oxygen pulse was given after the maximum velocity had been reached. An immediate 85% decrease of the reaction rate was observed. After consumption of the introduced oxygen the velocity increased again.

To obtain information about the nature and the amounts of the anaerobic reaction products, incubations were carried out with [U-^{14}C]-labelled 9-D- or 13-L-hydroperoxylinoleic acids respectively (Figs. 5 and 6).

Discussion

The experiments show that soybean lipoxygenase-1 can either form or destroy hydroperoxides, depending on the reaction conditions.

Hydroperoxidase activity is displayed even in the absence of an extra hydrogen donor but only occurs at a reasonable rate under strict anaerobicity. When oxygen is present in the system (>0.2 μM) the reaction is strongly inhibited, until, in a very slow reaction, the oxygen has been depleted. This accounts for the occurrence of a lag period. Thus, two types of reactions both leading to the conversion of hydroperoxides, can be distinguished.

Comparison of the thin-layer chromatography patterns of the anaerobic reaction products obtained from 13-L-[U-^{14}C]hydroperoxylinoleic acid as a substrate (Fig. 5) with those obtained in the anaerobic conversion of it in the presence of linoleic acid [7,8] shows the following: (a) dimeric compounds are absent; (b) a considerable amount of a substance (zone 3) is present, which behaves identically (R_F value, no carbonyl function) to the epoxy-hydroxyoctadecenoic acid as described by Garssen et al. [12]; (c) as in the anaerobic conversion in the presence of linoleic acid, 13-oxotridecadienoic acids (zone 4) and 13-oxo-octadecadienoic acid (zone 5) are formed, which are responsible for the absorption at 285 nm.

Calculating the percentage of the 13-oxotridecadienoic acid fragments (9c, 11t and 9t, 11t isomers) on a molar basis, a mean value of 23% ± 2 (S.D.) is found. From the radiochromatograms it is calculated that 42% ± 4 (S.D.) of the 13-L-hydroperoxylinoleic acid is converted into oxodienes. This value is in agreement with those obtained from the spectrophotometric data, 41% ± 2 (S.D.), taking into account the molar fractions of 13-oxotridecadienoic acid and 13-oxo-octadecadienoic acid as obtained from the radiochromatograms.

With 9-D-[U-^{14}C]hydroperoxylinoleic acid as substrate, a radiochromatogram is obtained, as presented in Fig. 6. In this case, apparently no chain cleavage reaction takes place. Only one oxodiene is formed (zone 7) which is tentatively identified as 9-oxooctadecadienoic acid. Also epoxy-hydroxyoctadecenoic acid (zone 4) seems to be present (no absorption >210 nm and no carbonyl function present).

Specific activities of the anaerobic hydroperoxide conversions by lipoxygenase-1 in the absence of linoleic acid are: 13-L-isomer : 5.0 nmol oxodiene $min^{-1} \cdot mg^{-1}$; 9-D-isomer : 91.0 nmol oxodiene $min^{-1} \cdot mg^{-1}$.

Linoleic acid stimulates the 13-L-hydroperoxide conversion 1500 fold, whereas it has no effect at all on the reaction rate of the 9-D-hydroperoxide conversion. This is in accordance with the observation by Garssen et al. [8], working at much lower enzyme concentrations, that virtually no conversion of the 9-D-isomer took place.

De Groot et al. [13] proposed a mechanism for the anaerobic conversion of 13-L-hydroperoxide in the presence of linoleic acid, in which the ferric enzyme is reduced by linoleic acid and the ferrous enzyme is in turn oxidized by the 13-L-hydroperoxide. In the latter step alkoxy radicals are formed, which give rise inter alia to oxodienes. Assuming that a similar mechanism is involved in the anaerobic conversion of 13-L-hydroperoxide in the absence of linoleic acid, reduction of the ferric enzyme must be performed by the 13-L-hydroperoxide. In that case, the formation of peroxy radicals has to be postulated. Apparently, the mechanism of the 9-D-hydroperoxide conversion must be different since no stimulation by linoleic acid is observed.

Work is in progress to gain further insight into the mechanism(s) of these reactions.

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