ON THE INTERACTION OF SOYBEAN LIPOXYGENASE-1 AND 13-L-HYDROPEROXYLINOLEIC ACID, INVOLVING YELLOW AND PURPLE COLOURED ENZYME SPECIES

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1. Introduction

Lipoxygenase-1 from soybeans (linoleate: oxygen oxidoreductase, EC 1.13.11.12, a dioxygenase containing non-heme iron) catalyses the conversion of linoleic acid and of other unsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene system into the corresponding conjugated (*n*-6)-L-hydroperoxy fatty acids under aerobic conditions [1].

Recently, we demonstrated by fluorescence- [2,3] EPR- [4] and ultraviolet absorption- [3,5] spectroscopy, that one equivalent of 13-L-hydroperoxy-linoleic acid (13-L-ROOH), converts native soybean lipoxygenase-1 into a species that is low fluorescent, contains Fe (III) and has an absorption maximum at 330 nm (ϵ_{330nm} = 1500 M^{-1} cm^{-1}).

Other evidence for a specific interaction of ROOH with the enzyme stems from earlier investigations on the kinetic lag phase of the aerobic lipoxygenase reaction [6–8]. Kinetic experiments by Smith and Lands [7] and Garssen [8] showed that the initial presence of more than a 100-fold molar excess of product hydroperoxide over enzyme (enzyme concentrations 0.03 and 0.01 μM respectively) is necessary to immediately attain the maximum reaction rate. These kinetic data suggest, that, besides the effect of one equivalent of hydroperoxide, another type of interaction between product and enzyme exists.

This paper presents spectroscopic and further kinetic evidence for the existence of an enzyme–hydroperoxide complex, which indeed differs from both the native enzyme, and the yellow Fe (III)-enzyme obtained after treatment with one equivalent of 13-L-ROOH.

2. Materials and methods

Lipoxygenase-1 was isolated from soybeans* according to Finazzi-Agro et al. [2]; spec. act. 190 μM O_2 min^{-1} mg^{-1}.

Linoleic acid was a gift from Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands (purity > 99%). 13- and 9- Hydroperoxylinoleic acids were prepared as described previously [9].

The free Fe (III) form of the enzyme was prepared by addition of 1.5 ml $1.4 \cdot 10^{-4}$ M 13-L-ROOH to 1.5 ml $2.27 \cdot 10^{-5}$ M lipoxygenase-1 at room temperature and pH 9.0 (0.2 M Tris–HCl), followed by filtration over a Sephadex-G25 (fine) column (40 cm × 1.2 cm), equilibrated with 0.05 M Tris–HCl buffer pH 9.0 at 4°C. The Fe (III) enzyme was characterized by its near-ultraviolet absorption spectrum (yellow colour, A_{max} = 330 nm; ϵ_{330nm} = 1500 M^{-1} cm^{-1}) and the EPR spectrum (signals at g = 7.5, 6.2, 5.9, 4.3 and 2.0).

Spectrophotometry was carried out with either a

* Registered Williams Soybeans, Lot T-431, obtained from Tabor Seed Division, 4248 West Main, Decatur, Illinois, USA.; January 1974.

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Beckman DB-GT grating spectrophotometer or a Unicam SP 1800 spectrophotometer.

EPR spectra were recorded on a Varian E-3 spectrometer with 100 Kcycles/s field modulation (cf. [10]) at a microwave power of 4mW. All measurements were carried out a 15 K, modulation amplitude of 10 G and at the same gain.

3. Results

The rate of formation of hydroperoxides from linoleic acid and molecular oxygen was measured for both the native enzyme (EPR-silent), and the yellow Fe (III) enzyme. The results (fig.1) show similar lag periods and progress curves for both forms of lipoxygenase-1. Therefore, it can be concluded that the conversion of the native enzyme into the Fe (III) form by the addition of one equivalent of

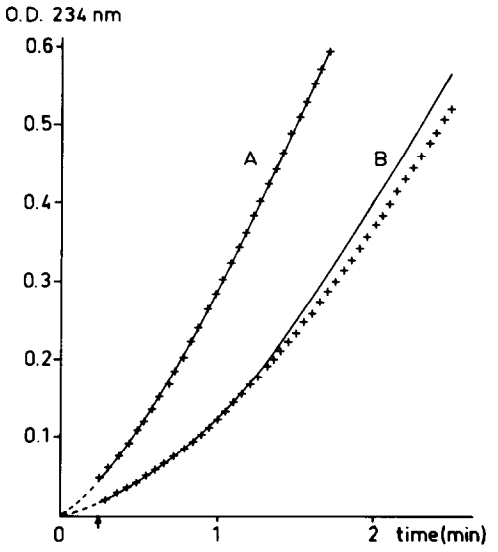

Fig.1. Progress curves showing the formation of 13-L-ROOH as a function of time, measured via continuous monitoring of the absorbance at 234 nm in a 1 cm cuvette. The reactions were started at t=o by adding 6 μl enzyme solution ($1.4 \cdot 10^{-6}$ M) to 3.5 ml reaction medium, containing $2.4 \cdot 10^{-4}$ M 0_2 and either $2.4 \cdot 10^{-4}$ M linoleic acid (A) or $4.8 \cdot 10^{-4}$ M linoleic acid (B). Measurements started 15–20 sec after the addition of enzyme (mixing time); the arrow indicated the start of the reaction traces: (——) for the native, EPR-silent form of the enzyme at t=o (+ + + +) for the Fe (III) form of the enzyme at t=o.

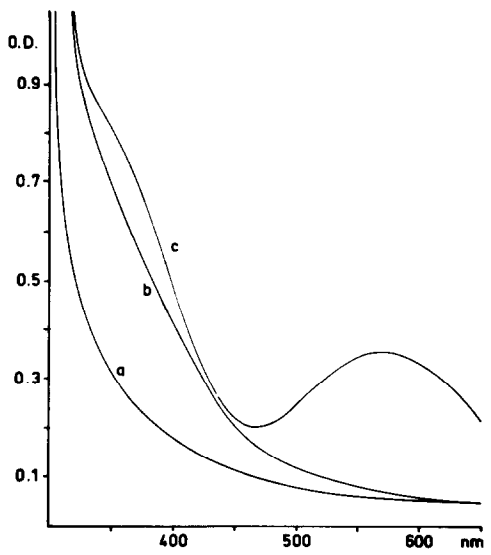

Fig.2. Effect of 13-L-ROOH on the ultraviolet and visible absorption spectrum under aerobic conditions at 5°C and pH 9.0. (a) 0.38 mM Lipoxygenase solution (38 mg/ml). (b) Reaction mixture of 400 μl of a lipoxygenase solution (38 mg/ml) and 84 μl of a hydroperoxide solution (2 mM). Final concentrations: 0.31 mM and 0.34 mM respectively. (c) Reaction mixture of 400 μl of a lipoxygenase solution (38 mg/ml) and 284 μl of a hydroperoxide solution (2 mM). Final concentrations: 0.22 mM and 0.83 mM respectively.

13-L-ROOH with respect to the enzyme is not the only step in the activation of the enzyme. Previous kinetic experiments showed the necessity of a large excess of hydroperoxide over enzyme to abolish the lag phase. Therefore we established the EPR and optical spectroscopic parameters of concentrated enzyme solutions to which increasing amounts of hydroperoxylinoleic acids were added.

3.1. *Optical and EPR spectra of lipoxygenase-1*

The optical spectrum of native lipoxygenase-1 shows no significant features other than the protein absorption at 280 nm [11] (fig.2a). The EPR spectrum shows a low signal at g = 4.3 and a complex signal around g = 2 (fig.3a). These signals stem from contaminating iron, manganese and copper [4].

The addition of a stoichiometric amount of 13-L-HOOH to lipoxygenase causes the colourless solution to turn into yellow [5], with a corresponding

absorption maximum (fig.2b) at 330 nm ($\epsilon_{330\ nm}$ = 1500 M^{-1} cm^{-1}). The EPR spectrum shows new signals at g = 7.5, 6.2, 5.9, 4.3 and 2.0 (fig.3b).

Upon a further addition of a 3-fold molar excess of 13-L-ROOH, the yellow colour of the solution turns into purple and the absorption spectrum now shows an additional absorption band with a maximum at 570 nm ($\epsilon_{570\ nm}$ = 10^3 M^{-1} cm^{-1}) and a slight increase of the absorption at 330 nm (fig.2c). The EPR spectrum of the purple solution is strikingly different from that of the yellow one; the intensities of the signals at g = 7.5, 6.2 and 5.9 are greatly reduced, whereas the signal at g = 4.3 is strongly increased. (fig.3c).

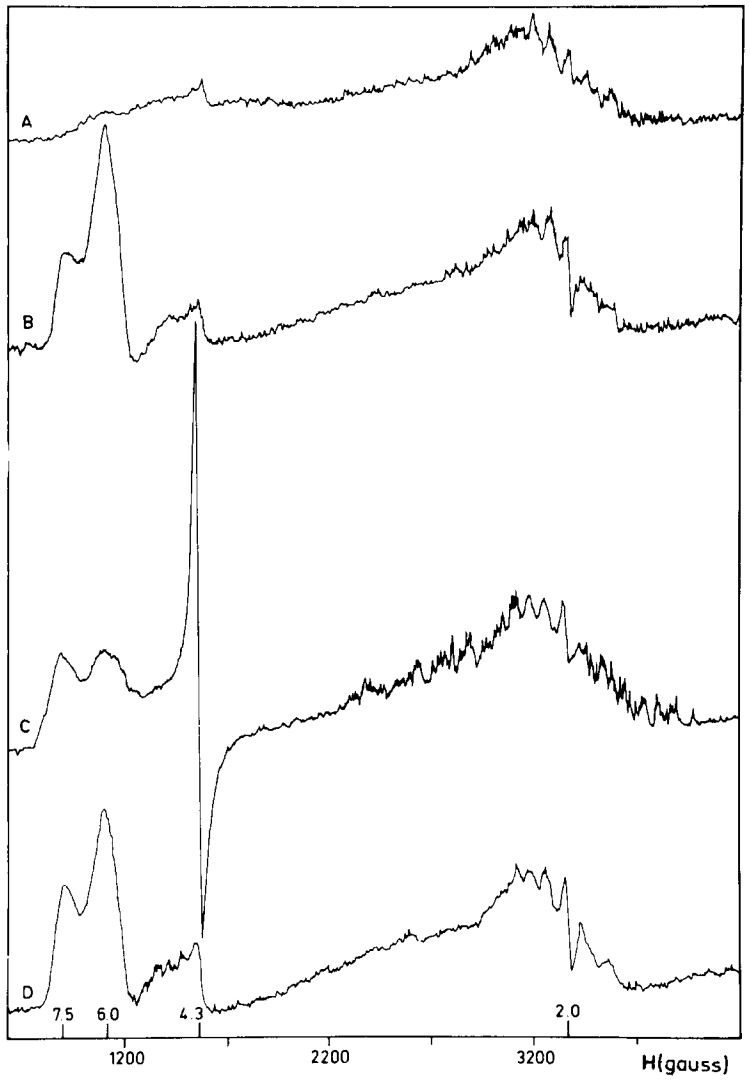

Fig.3. Effect of 13-L-ROOH on the EPR spectrum of lipoxygenase-1. (A) 300 μl of a lipoxygenase solution (30 mg/ml). (B) 8 μl of a hydroperoxide solution (11.4 mM) was added to 300 μl of a lipoxygenase solution (30 mg/ml). Final concentrations: 0.29 mM and 0.30 mM. (C) another 16 μl of 11.4 mM hydroperoxide solution was added to the reaction mixture. Final concentrations: 0.28 mM and 0.84 respectively. (D) the same reaction mixture as described under (C) after 1 hr at 0°C. Microwave frequency, 9.307 GHz.

On standing (10 min at room temperature or 1 hr at 0°C) the sample gradually looses its purple colour and becomes yellow again. The yellow species derived from the purple one shows optical and EPR spectra, which are identical to those of the yellow species obtained after treatment of the native enzyme with one equivalent of 13-L-ROOH.

The yellow sample formed from the purple one can be reconverted into the purple species by the addition of another amount of 13-L-ROOH. This procedure can be repeated several times.

In the absence of oxygen the same results with 13-L-ROOH are obtained, although the relative intensities of the signals near g = 6 show some differences as described before [4].

During the purple to yellow transition the hydroperoxide is converted into mainly (*trans*-12, 13-epoxy)-11-hydroxy-*cis*-octadec-9-enoic acid [12].

The yellow and purple species can also be formed when, instead of 13-L-ROOH, comparable amounts of linoleic acid are added aerobically, obviously this has to be ascirbed to the formation of 13-L-ROOH.

It should be noted, that 9-D-hydroperoxylinoleic acid is incapable of forming the purple species.

4. Discussion

The results presented above show, that three spectroscopically different forms of soybean lipoxygenase-1 can be distinguished. (a) The native enzyme. (b) The yellow species, which is formed upon the addition of a stoichiometric amount of 13-L-ROOH to the native enzyme. (c) The purple species, which arises, when an excess (3 to 5 molar fold) of 13-L-ROOH is added to the native enzyme or to the yellow species.

Both coloured enzyme forms contain iron in the ferric state, although the corresponding EPR spectra demonstrate that the ligand symmetries are different. The purple species is a complex of the yellow enzyme with 13-L-ROOH. These conclusions can be drawn on the bases of lability of the purple complex.

The purple–yellow transition is accompanied by a conversion of the hydroperoxide into (*trans*-12, 13-epoxy)-11-hydroxy-*cis*-octadec-9-enoic acid. This compound is always formed in minor amounts upon normal aerobic incubations of linoleic acid and lipoxygenase. This observation makes it probable, that under usual reaction conditions the coloured enzyme species play a role in the oxygenation reaction.

The native and yellow enzyme species are kinetically indistinguishable (cf. fig.1): both species have the same kinetic lag phase, which can be abolished by addition of 13-L-ROOH. Therefore binding of 13-L-ROOH to the enzyme seems to be essential as is the presence of iron in the Fe (III) state in the function of the enzyme as electron acceptor. The purple enzyme species meet the latter two requirements, which make it tempting to suggest, that the purple species is the active one during the aerobic reaction of lipoxygenase. However, to gain further insight in the activation of native lipoxygenase by 13-L-ROOH kinetic experiments about the rate of formation of enzyme–13-L-ROOH complexes are indispensable.

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