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LIPOXYGENASES, PROPERTIES AND MODE OF ACTION

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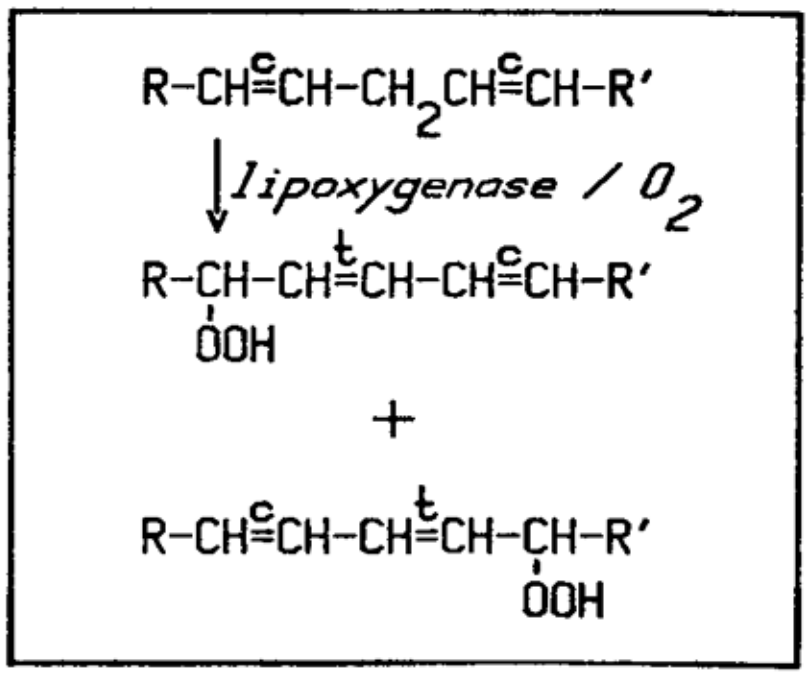
I. INTRODUCTION

Lipoxygenases (linoleate:oxygen oxidoreductase; EC 1.13.11.12) are widely distributed in nature and have been found in both the plant and the animal kingdom. Historically, representatives of this type of enzymes were first characterized from plant sources, in particular from soybeans and other *Leguminosae*. Over the years, the catalytic properties of lipoxygenases have been intensively studied because of their possible role in the oxidative breakdown of unsaturated lipids in foods and edible oils during processing and storage (1,2).

Lipoxygenases catalyse the formation of hydroperoxides from unsaturated lipids containing one or more 1,4-*cis,cis*-pentadiene functions (Scheme 1).



Scheme 1. Formation of hydroperoxides from unsaturated fatty acids. For linoleic acid R= CH_3-$(CH_2)_4$-; R'= -$(CH_2)_7$-COOH .

In addition to the formation of hydroperoxides some lipoxygenases are also capable of catalyzing subsequent steps in the metabolism of unsaturated fatty

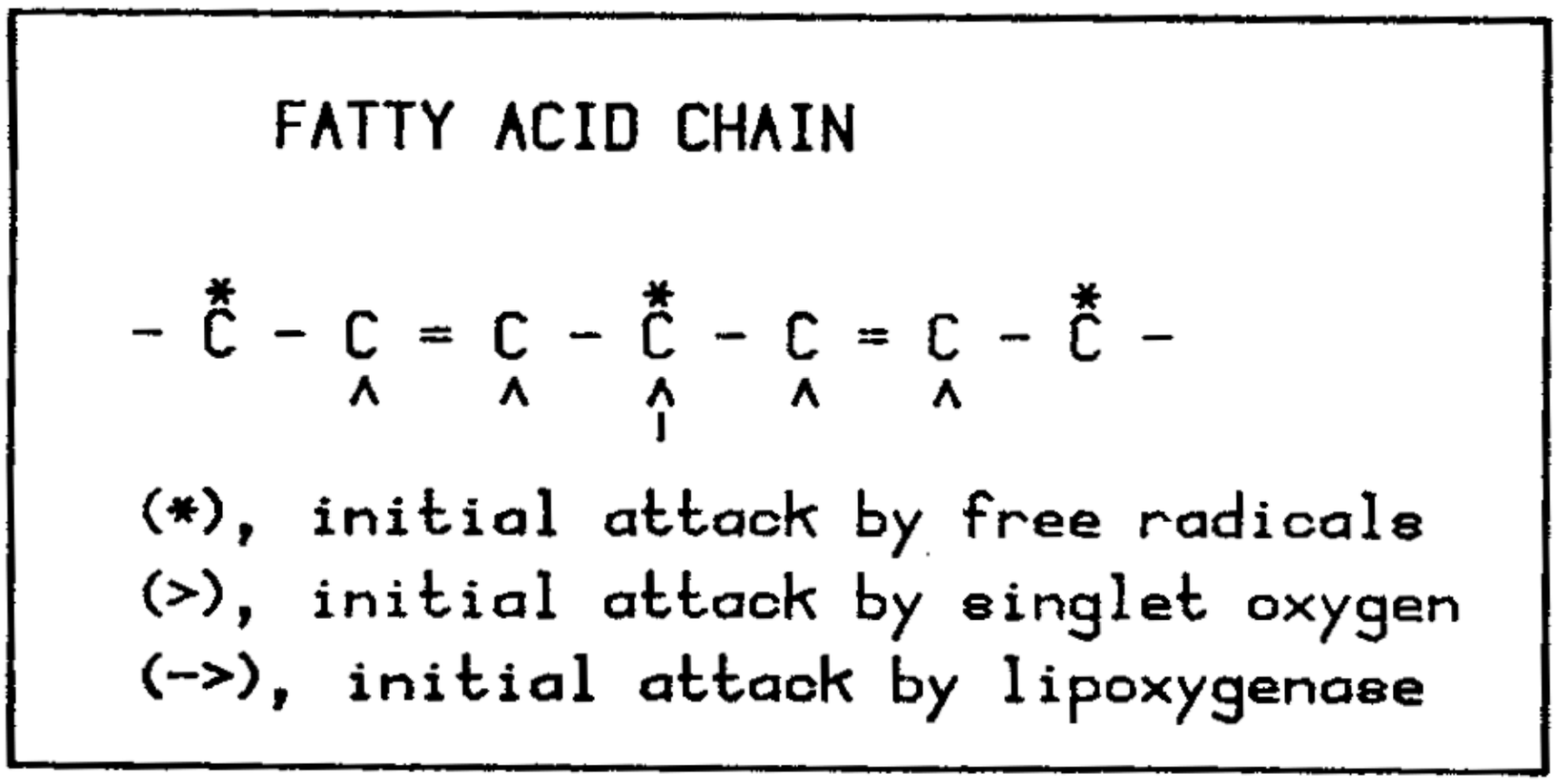
acids e.g. the transformation of fatty acid hydroperoxides into alkanes, ketones, aldehydes, epoxides, and other derivatives.

Lipoxygenases known sofar have molecular masses ranging from 60000 to 100000 and contain one non-heme iron center that is essential to the proper catalytic functioning of the enzyme.

The present paper summarizes some recent results in the field of lipoxygenase research with particular emphasis on those obtained with enzymes from plant origin.

II. FORMATION OF UNSATURATED FATTY ACID HYDROPEROXIDES

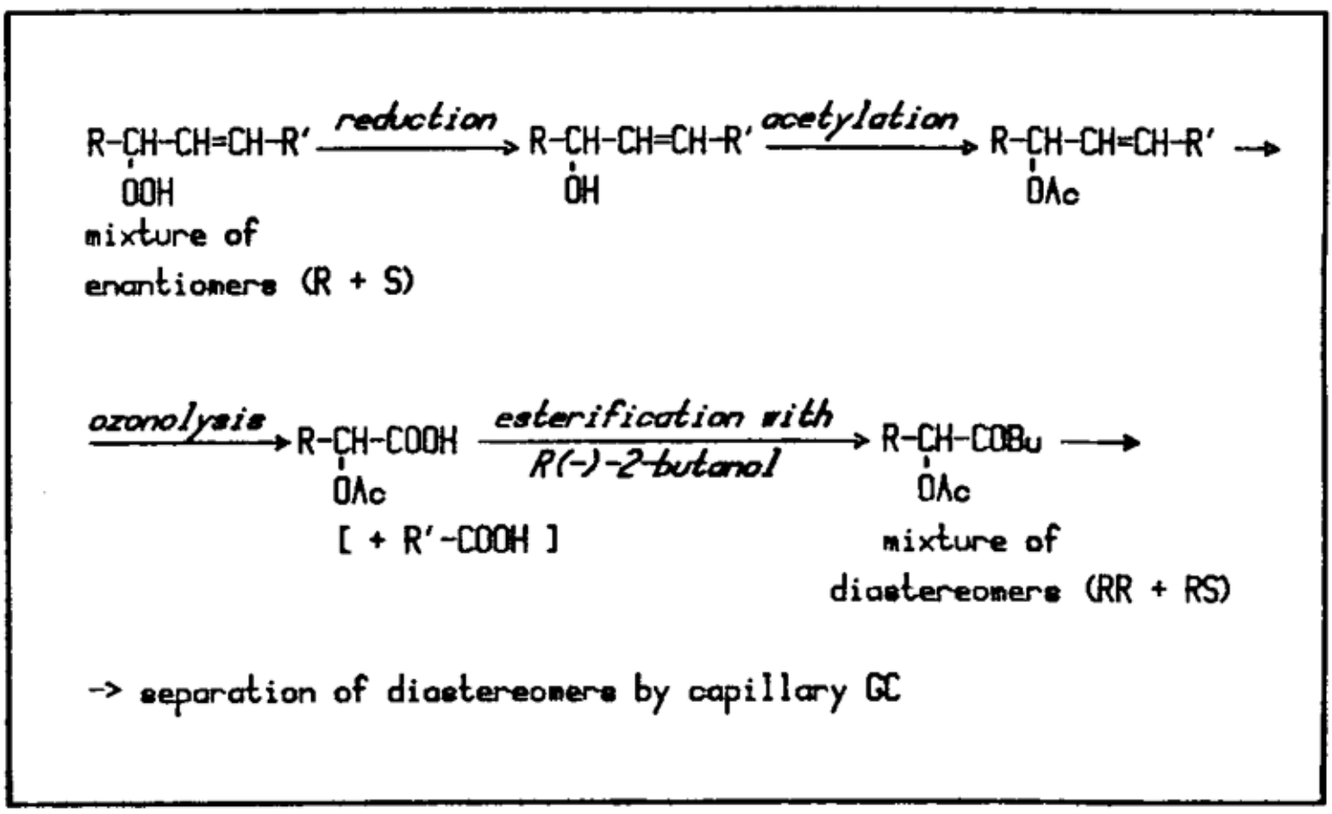
Hydroperoxides can be formed from unsaturated fatty acids and molecular oxygen through three different processes:
1) free-radical autoxidation; 2) attack of singlet molecular oxygen and 3) lipoxygenase-catalysis.
Once the fatty acid hydroperoxides have been properly analysed it can, in principle, be deduced which of the above processes has controlled the hydroperoxide-formation. The free-radical autoxidation shows a slight regioselectivity with regard to the carbon radical that is oxygenated but does hardly favor the formation of any particular enantiomer at such a carbon free radical (3,4). The primary attack of (activated) singlet molecular oxygen is directed towards the π-electrons of the double bonds of the fatty acid chain rather than at the methylene groups either between or next to the double bonds as is the case in a free radical process. This leads to the formation of a number of positional isomers, some of which can only be formed through the singlet oxygen pathway, namely the non-conjugated products (Scheme 2).



Scheme 2. Initial steps in enzymic and non-enzymic formation of hydroperoxides

Hydroperoxide formation involving lipoxygenase starts by abstracting in a stereospecific way one of the hydrogens from the central methylene group of a 1,4-*cis,cis*-pentadiene system. The electrons of the pentadienyl radical then rearrange to form one *trans* double bond thereby locating the unpaired electron at one of the terminal carbon atoms to which subsequently ground state (triplet) oxygen is attached in a stereospecific way. When a fatty acid chain contains multiple 1,4-pentadiene systems (e.g. arachidonic acid) lipoxygenase also performs multiple dioxygenations.

This often leads to complex mixtures of geometric and stereo-isomers. Mixtures of geometric isomers can conveniently be resolved by high-performance liquid chromatography (HPLC). A full stereochemical characterization of the mono- and dihydroperoxy fatty acids requires a number of steps that are summarized in Scheme 3 (5).



Scheme 3. Determination of enantiomeric compositions by chiral derivatization and capillary gas liquid chromatography, Ac= CH_3CO

This procedure has been applied to the hydroperoxides formed from a variety of unsaturated fatty acids, the results of which are compiled in Table 1.

These analyses clearly show the sharp specificity of lipoxygenase to produce hydroperoxides with the S-configuration, soybean lipoxygenase-2 at pH 9.0 being the exception. However, it is interesting to note that the cyclo-oxygenase from sheep vesicular glands also forms predominantly 13(S)- and 9(R)-HOD from linoleic acid (6). In a number of cases it has also been determined which of the prochiral hydrogen atoms is removed from the central methylene group in the process of hydroperoxide formation. It then appears that, provided the pentadiene system is

oriented as a planar entity, hydrogen abstraction and oxygen insertion occur antarafacially (6-8).

TABLE 1
STEREOCHEMISTRY OF HYDROPEROXIDE FORMATION FROM VARIOUS SUBSTRATES*

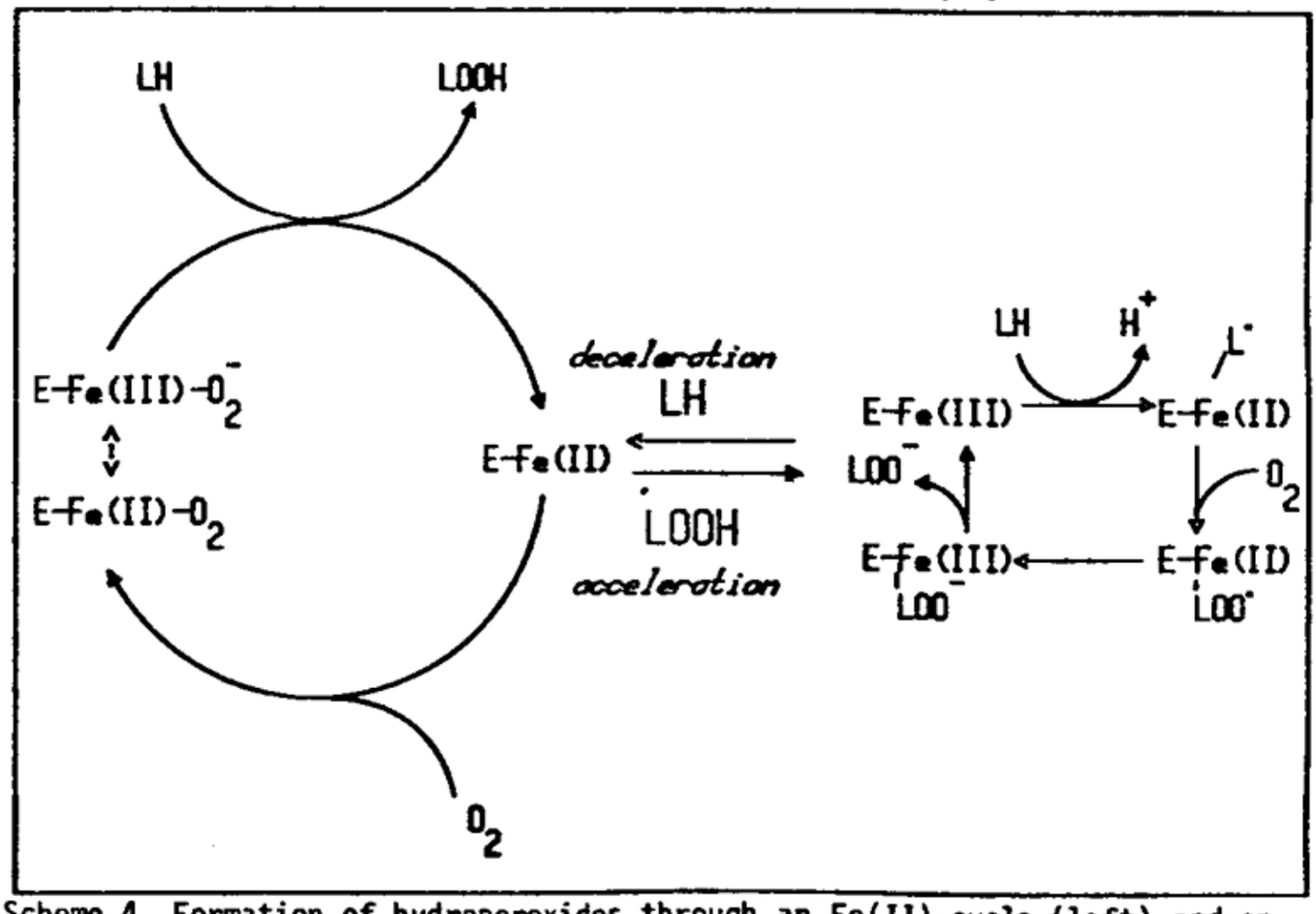
Reaction product	Enzyme (pH)	Ozonolysis product	Absolute configuration assignment of parent HPFA
13HPOD	soybean-1 (9.0)	2-OH-heptonoic acid	R : S = 2 : 98
9HPOD	corn germs (6.6)	2-OH-sebacic acid	R : S = 6 : 94
9HPOD	soybean-2 (9.0)	2-OH-sebacic acid	R : S = 79 : 21
13HPOT	soybean-1 (9.0)	malic acid	R : S = 7 : 93
15HPETE	soybean-1 (9.0)	2-OH-heptanoic acid	R : S = 4 : 96
8,15DHPETE	soybean-1 (8.7)	2-OH-heptanoic acid, malic acid	R : S = 6 : 94 R : S = 5 : 95
5,15DHPETE	soybean-1 (8.7)	2-OH-heptanoic acid, 2-OH-adipic acid	R : S = 4 : 96 R : S = 10 : 90

HP, hydroperoxide or hydroperoxy
FA, fatty acid
OD, octadecadienoic acid (linoleic acid)
OT, octadecatreinoic acid ([alfa]linolenic acid)
ETE, icosatetraenoic acid (arachidonic acid)
*) reaction temperature 4°C

III. KINETIC ASPECTS OF THE MECHANISM OF HYDROPEROXIDE FORMATION

Soybean lipoxygenase-1 very efficiently catalyzes the formation of hydroperoxides from suitable substrates like linoleic acid. However, some reaction conditions are critical, in particular the concentrations of the primary substrates fatty acid and oxygen, pH and the concentration of the product hydroperoxide. As early as 1958 Haining and Axelrod (9) reported on the activating effect of product hydroperoxide on the rate of the reaction. At relatively high substrate concentrations a kinetic lag phase was observed which could effectively be abolished by the addition of product hydroperoxide. The length of such a kinetic lag phase was found to be specifically dependent on the nature and the amount of the hydroperoxide in the reaction medium. Recently, Funk et al. (10) have reinvestigated this phenomenon and showed that only those product analogues

having an intact hydroperoxide group displayed the activating effect on the reaction rate. In principle, several ways are open to explain non-linearity in the initial parts of progress curves. As for lipoxygenase, a concept of two enzymes with different turnover rates and occurring in a varying ratio in the initial stage of the reaction, has been put forward by Vliegenthart and Veldink (11). At the start of a reaction sequence the enzyme is in a ferrous state (with one of the iron ligands presumably being dioxygen) and as such it probably is a comparatively poor catalyst of hydroperoxide formation. However, as soon as hydroperoxide is being produced by the so-called ferrous-cycle, some fraction of the enzyme molecules is converted by the hydroperoxide just formed into a ferric species which presumably constitutes a much more efficient catalyst. In this view a steady-state will be reached at a constant ratio of the contributions of the Fe(II)- and Fe(III)-cycles. The relative amounts of Fe(II)- and Fe(III)--species are thought to be directly dependent on the substrate and product concentrations which implies that the occurrence of a lag phase will not depend on the initial oxidation state of the enzyme at the very beginning of the experiment. Any given amount of lipoxygenase that has been oxidized to an Fe(III)-state prior to a kinetic experiment will produce a progress curve which is identical to the one obtained with the same amount of the enzyme in its native state (12,13). Scheme 4 summarizes the interplay of Fe(II)- and Fe(III)-cycles.



Scheme 4. Formation of hydroperoxides through an Fe(II)-cycle (left) and an Fe(III)-cycle (right)

Another class of hydroperoxide formation comprises multiple dioxygenations of fatty acids. Here, more than one molecule of dioxygen is inserted in those substrates that contain multiple 1,4-*cis*,*cis*-pentadiene systems. Bild et al. (14,15) reported the stepwise conversion of arachidonic acid (ETE) into 15HPETE which is in turn dioxygenated to yield 8,15DHPETE. It was subsequently demonstratedby Van Os et al. (16) that in this second step the 15HPETE is in fact converted into a mixture of 8,15HPETE (60%) and 5,15HPETE (40%). The rates and the Km-values of the first and the second oxygenation steps were found to differ significantly (Table 2).

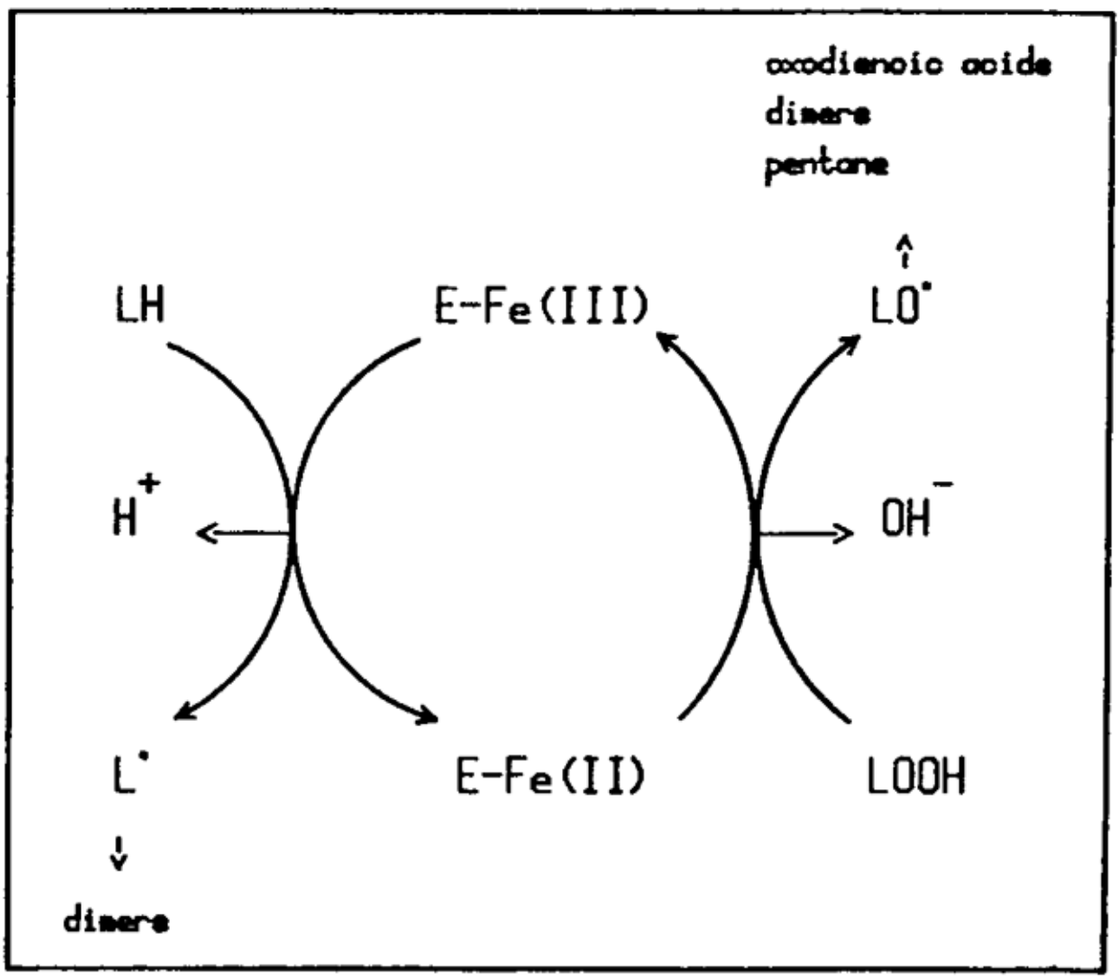
TABLE 2
KINETIC PARAMETERS OF THE DIOXYGENATION STEPS OF ARACHIDONIC ACID (ETE)

	step 1 [ETE → 15HPETE]	step 2 [15HPETE → 8,15HPETE + 5,15HPETE]
Km	8.5 μM, pH 10	440 μM, pH 8.7
k(cat)	225 s^{-1}	25 s^{-1}

IV. CONVERSION OF HYDROPEROXIDES BY LIPOXYGENASE

Besides being the primary product of the lipoxygenase reaction unsaturated fatty acid hydroperoxides may also be the substrate for this type of enzyme. A well-known example of this type of reaction occurs anaerobically between soybean lipoxygenase-1, linoleic acid and hydroperoxylinoleic acid. The reaction has been studied by Garssen et al. (17,18) and by Verhagen et al. (19) and leads in an enzymically controlled stoichiometry to a variety of products. The proposed mechanism bears a strong resemblance to the Fe(III)-cycle of the aerobic reaction of hydroperoxide formation. However, as oxygen is virtually absent under these conditions the hydroperoxide is thought to react with the Fe(II)-enzyme instead, which leads to an Fe(III)-species, an alkoxy radical and a hydroxyl-ion (Scheme 5).

The kinetics of the reaction (19) support the idea of a single binding site on the enzyme that alternatingly binds the fatty acid hydroperoxide or the fatty acid (substituted enzyme or "ping-pong" mechanism). The reaction is inhibited by high concentrations of both the fatty acid and the fatty acid hydroperoxide.



Scheme 5. Anaerobic conversion of unsaturated FA and HPFA by lipoxygenase

Although, for obvious reasons, termed an "anaerobic" reaction this mode of hydroperoxide conversion need not be confined to completely anaerobic systems. Several authors (20,21), using different lipoxygenases, have reported the production of compounds under apparently aerobic conditions that are formed with lipoxygenase-1 only when oxygen is absent.

Interestingly, a lipoxygenase from rabbit reticulocytes, which is akin to soybean lipoxygenase-1 in many other respects, also shows this type of anaerobic conversion of hydroperoxides (22).

V. SPECTROSCOPIC CHARACTERISTICS OF LIPOXYGENASE

Lipoxygenase-1 in its resting state is a colourless protein that turns yellow by reacting with one molar equivalent of 13HPOD (23). Through the same reaction also the electron paramagnetic resonance (epr) spectrum changes: the resting enzyme is virtually epr-silent whereas the yellow form displays a complex signal around g6. This yellow, Fe(III)-containing species is considered to be a key intermediate in both the aerobic and anaerobic reaction. A detailed analysis of this unique type of epr-signal shows that it is most probably built up from at

least three high-spin Fe(III)-species (24). An excellent correlation was found between the formation of the yellow colour and the appearance of the g6 epr--signal (Fig. 1B). When either native or yellow lipoxygenase-1 is allowed to react with a molar excess of 13HPOD it turns purple, while the epr spectrum now shows an additional resonance line at g4.3. This purple species is regarded as a complex between the yellow species and 13HPOD. As with the yellow species a linear relationship is found between the appearance of the colour and the amount of epr-visible iron at g4.3 (Fig. 1A). The total amounts of epr-visible iron in the yellow and purple species are above 75%.

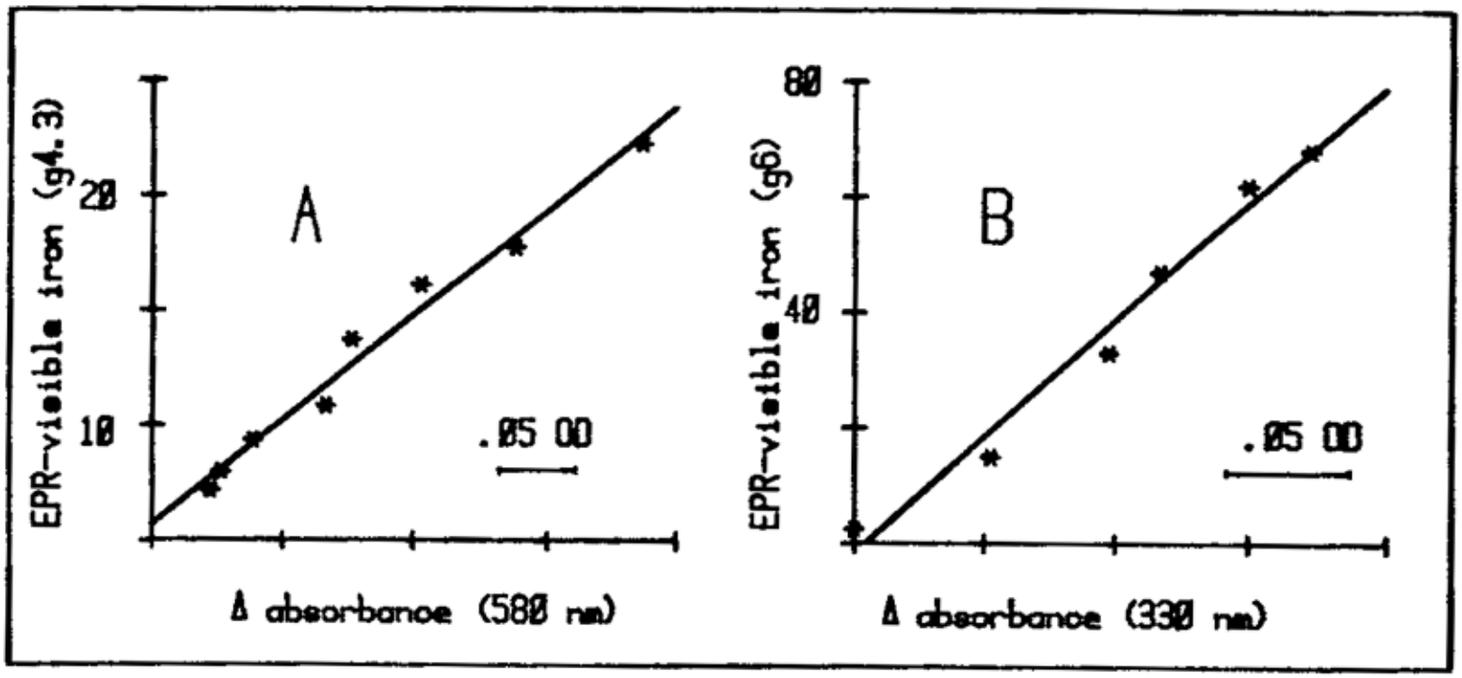


Fig. 1. Correlations between the amounts of iron visible in the EPR-signals around g4.3 (A) and g6 (B) and colour development in the protein

VI. IMMUNOLOGICAL CLASSIFICATION OF LIPOXYGENASES

In double gel immunodiffusion tests lipoxygenases from a number of plant species were investigated to see if cross-reactions occurred with antisera directed against lipoxygenases-1 and -2 from soybeans.

Cross-reactions were found only with lipoxygenases isolated from members of the *Leguminosae* family, whereas no specific precipitation could be detected with species from *Gramineae*, *Linaceae* and *Solanaceae*. Table 3 contains the results of such comparative tests.

TABLE 3

IMMUNOLOGICAL CROSS-REACTIONS OF LIPOXYGENASES WITH ANTISERA DIRECTED AGAINST SOYBEAN LIPOXYGENASES-1 AND -2

Enzyme/source	Precipitation* with antiserum against: LIPOXYGENASE-1	LIPOXYGENASE-2
soybean lipoxygenase-1	+	-
soybean lipoxygenase-2	-	+
Glycine max (L.) Merr. (soybean)	+	+
Pisum sativum L. (green pea)	-	+
Phaseolus vulgaris L. (red kidney bean)	-	+
Zea mays L. (maize)	-	-
Triticum aestivum L. (wheat germs)	-	-
Linum usitatissimum L. (flax)	-	-
Solanum melongena L. (eggplant)	-	-
Solanum tuberosum L. (potato)	-	-

*) Precipitation lines fusing with a reference line were considered as a positive reaction.

These results indicate that the presence of structural similarities with lipoxygenases-1 and -2 only occurs in other members of the *Leguminosae* family. It is noteworthy that sofar, lipoxygenases from *Leguminosae*-species are the only enzymes from plant origin capable of producing hydroperoxides with the R-configuration, namely soybean lipoxygenase-2 (pH 9.0) and pea lipoxygenase (pH 9.0) (25). Future work should reveal whether or not this property is confined to members of this plant family.

ACKNOWLEDGEMENTS

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