CHAPTER 2

Lipoxygenases

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I. INTRODUCTION

Lipoxygenases (EC 1.13.11.12) are defined as enzymes that catalyze the oxgenation of unsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene system. Enzymes from plant origin produce cis,trans-conjugated monohydroperoxides as primary products (Scheme 1), but may show significant differences in regio- and/or stereospecificities.

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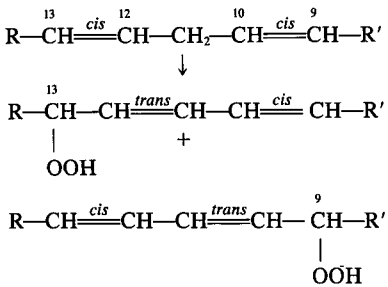


Scheme 1. Oxygenation of polyunsaturated fatty acids by lipoxygenase.

It has been found that besides the dioxygenase activity of lipoxygenases, several of these enzymes show an interesting catalytic activity under anaerobic conditions. The anaerobic activity of soybean lipoxygenase-1 is the best investigated example of this type of reaction. Here, the product hydroperoxide serves as the second substrate instead of oxygen. Although the proposed mechanism of this reaction is elegantly simple, a complex pattern of products is formed, i.e., fatty acid dimers (with and without oxygen functions), oxodienoic acids, and *n*-pentane (see Section VIII). The lipoxygenases from animal origin differ in many respects from those of plant origin; a common feature is the presence of cis,trans-conjugated double bonds in the product hydroperoxide. However, in most cases the isolated end product contains a hydroxy rather than a hydroperoxy group. So far, no anaerobic activity of this type of enzyme has been reported.

In this chapter, we emphasize recent developments in the chemistry and biochemistry of plant lipoxygenases.

II. LIPOXYGENASE ASSAYS

A. Aerobic Reaction

Several possibilities exist for the determination of lipoxygenase activity. The consumption of oxygen can be measured by polarography or manometry. Knowledge of the precise stoichiometry of the reaction is a prerequisite of these methods. The chemical or spectroscopic characteristics of the product may also be used for this purpose. The absorbance of the cis,trans-conjugated double bond system at 234 nm is a convenient parameter. In principle, the formation of the hydroperoxy

group can be determined colorimetrically in a discontinuous way. Recently, a spin-reduction assay for lipoxygenase activity has been proposed [*1*] that was found to be particularly useful in turbid systems. The variation in pH optima and substrate specificities of the lipoxygenases make it necessary to select the most suitable assay conditions for each enzyme. Generally, a relatively high concentration of linoleic acid (1.8 m*M*) is used. Since this concentration exceeds by far the critical micelle concentration of linoleic acid at any pH, erroneous results may be obtained [*2–4*]. Furthermore, one should avoid using substrate concentrations above the initial oxygen concentration (i.e., 240 μ*M* at room temperature) because eventual depletion of oxygen can give rise to the formation of products that are characteristic of the so-called anaerobic reaction [*5*]. Another reason for using lower substrate concentrations lies in the fact that several lipoxygenases show a remarkable type of substrate inhibition. In aqueous solutions, lipids tend to form multimolecular aggregates. This is demonstrated in Fig. 1 for mixtures of linoleic acid and hydroperoxylinoleic acid [*4*].

It should be noted further that the presence of hydroperoxides has little influence on aggregation behavior. For enzymes which are known to have apparent pH optima at pH 6–7, it is unknown whether the enzyme utilizes monomers and/or aggregates up to true micelles. Enzymes other than soybean lipoxygenase-1 often exhibit oxygenating activities at pH values in the range 8–10, provided that very low substrate concentrations

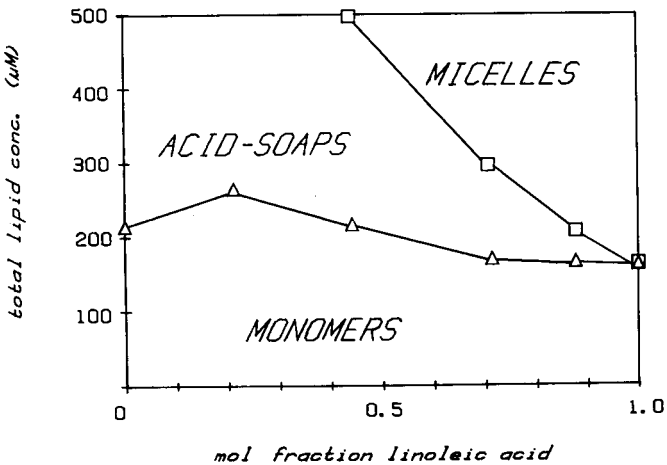


Fig. 1. Concentration ranges of monomers, acid soaps, and micelles in mixtures of linoleic acid and 13-L-hydroperoxylinoleic acid. (0.1 *M* sodium borate buffer, pH 10, 23°C).

are used ($\leqslant$50 μM) because substrate inhibition is rather strong. Heath and Tappel [*6*] have described a routine assay for the measurement of hydroperoxides which is based on the reduction of hydroperoxides (including fatty acid hydroperoxides) by the enzyme glutathione (GSH) peroxidase (GSH:H_2O_2 oxidoreductase; EC 1.11.1.9) [*7,8*]. Subsequently, the oxidized glutathione (GSSG) is reduced by NADPH (monitored spectrophotometrically) and glutathione reductase (EC 1.6.4.2). This coupled assay method is also suited to measure hydrogen peroxide. Hydroperoxides can be detected in concentrations down to 3 nmol/ml. Grossman and Zakut [*9*] have recently reviewed the assay methods for the aerobic lipoxygenase activity.

B. Anaerobic Reaction

A peroxidase activity of lipoxygenase-1 has been found under anaerobic conditions by Garssen *et al.* [*5*]. A complex pattern of reaction products is formed [*10*] in which a 1:1 mixture of 13-oxooctadeca-9,11-dienoic acid and 13-oxotrideca-9,11-dienoic acid provides a convenient parameter for a spectrophotometric assay. Any enzymically formed polyunsaturated fatty acid hydroperoxide will be converted into this type of oxodienoic acids with an absorption maximum at 285 nm with a molar absorption coefficient of 25,000/$M \cdot cm^{-1}$. In a typical experiment the cuvette should be oxygen-free achieved by flushing it with pure argon for several minutes. The assay mixture should also contain 13-L-hydroperoxylinoleic acid and linoleic acid which can be injected into the cuvette through the sealed cap. Likewise, an aliquot of the enzyme preparation under investigation is introduced and the change in the absorbance at 285 nm is then recorded [*11*]. Both linoleic acid and hydroperoxylinoleic acid can cause substrate inhibition which should be taken into account when evaluating kinetic results.

The anaerobic activity of lipoxygenase can also be determined from the bleaching of suitable dyes, e.g., 2,6-dichlorophenolindophenol (DCPIP) [*12*]. DCPIP appears not to be bleached during the aerobic phase of the reaction. Obviously, the bleaching process is initiated by free radicals produced during the anaerobic reaction of lipoxygenase with linoleic and hydroperoxylinoleic acids. As yet, the nature of the reaction products has not been determined.

III. ISOLATION AND PURIFICATION

A procedure for the isolation and purification of soybean lipoxygenase-1 has been described by Finazzi-Agrò *et al.* [*13*]. In order to reduce the amounts of adherent nonessential metals like Mn and Cu, Galpin *et al.*

[*14*] have introduced the use of metal chelators in all the purification stages. The general procedure is summarized in the following steps: After the beans have been swollen in demineralized water and ground in 0.1 *M* acetate buffer (pH 3.9) the mixture is centrifuged and the resultant supernatant stirred with CM-Sephadex C-50. The adsorbed proteins including lipoxygenases are desorbed by elution with a linear (0–0.5 *M*) NaCl gradient in 0.1 *M* acetate buffer (pH 5.5). The enzyme preparation is then brought to 60% saturation with solid ammonium sulfate and further purified by ion exchange chromatography over DEAE-Sephadex A-50 and CM-Sephadex C-50. From 1 kg of dry soybeans, 1 g of lipoxygenase-1 can be obtained with a specific activity of 240 μmol/min/mg in a standard polarographic assay (1.8 m*M* ammonium linoleate in .1 *M* sodium borate buffer, pH 9.0).

IV. AMINO ACID COMPOSITION

Several reports are available on the amino acid composition of soybean lipoxygenase-1 (cf. [*15–19*]). A recent analysis carried out by Spaapen [*20*] is given in Table I.

TABLE I Amino Acid Composition of Soybean Lipoxygenase-1

Amino acid residue	Number of residues
Asp	87
Thr	44[a]
Ser	52[a]
Glu	111
Pro	45
Gly	66
Ala	66
half-Cys	5
Val	52[b]
Met	17
Ile	50[b]
Leu	82
Tyr	43[a]
Phe	36
Lys	52
His	28
Arg	36
Trp	11
Total	883

[a] Values determined by extrapolation to zero hydrolysis time.
[b] Values determined by extrapolation to infinite hydrolysis time.

As noted before, the number of half-cystine residues shows a relatively large variation. Based on a molecular weight of 100,000 Stevens *et al.* [*19*] found four cysteines plus two disulfide groups and Schroeder [*18*] reported the presence of five half-cystines. In a reinvestigation Spaapen *et al.* [*21*] found five half-cystines in the form of cysteic acid residues in the amino acid analysis (Table I). After denaturation of the enzyme with 1% sodium dodecylsulfate or 6 *M* guanidine HCl, the presence of five free thiol groups could be demonstrated with three different SH reagents (Table II).

Therefore, it can be concluded that soybean lipoxygenase-1 contains no cystine residues. In the absence of chaotropic agents, three thiol groups can easily be modified with organic mercuric halides [*21*]. This was derived from experiments with ^{14}C-labeled methylmercuric iodide. Interestingly, modification of the native enzyme with these reagents affords significant alterations in enzymic activity and in catalytic properties. The N-terminus of the polypeptide is probably blocked, since no terminal amino acid could be demonstrated by the Edman degradation (L. J. M. Spaapen, unpublished results).

V. COFACTORS

Lipoxygenase-1 is a metalloenzyme having nonheme iron as a prosthetic group. To date, most of its physical characteristics appear unique, making a comparison with other iron-containing proteins virtually impossible. It has been established that lipoxygenases from several sources contain 1 mole of iron. Iron is directly bound to the polypeptide back-

TABLE II Number of Sulfhydryl Groups in Soybean Lipoxygenase-1

Sulfhydryl reagent[a]	pH	Denaturing agent	Number of SH groups
DTNB	8.0	1% SDS	4.7
DTNB	8.0	6 *M* guanidine HCl	4.7
4-PDS	5.0	6 *M* guanidine HCl	5
4-PDS	7.5	6 *M* guanidine HCl	4.7
4-PDS	7.5	1% SDS	5
*p*CMB	7.0	1% SDS	4.7

[a] Abbreviations: *p*CMB, *p*-Chloromercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 4-PDS, 4,4′-dithiopyridine; SDS, sodium dodecylsulfate.

bone, but little is known about the actual coordination sphere. In either a ferric or a ferrous state it is the only nonprotein component of lipoxygenase known to play a clearly defined role in the catalytic mechanism. Iron chelators like bipyridyl and *O*-phenanthroline will not very readily remove the iron from the protein. Efficient binding of the metal by such chelators only occurs in the presence of 2% sodium dodecylsulfate (SDS) or 6 *M* guanidine HCl. This indicates that the iron of soybean lipoxygenase-1 is tightly bound to the protein core.

The activity of a lipoxygenase isolated from human platelets was found to depend on the presence of Fe(III) [*22*]. A very pronounced inhibition was observed in the presence of chelating agents like EDTA and *O*-phenanthroline. This inhibition could be reversed by Fe(III) but not by Fe(II).

A more comprehensive analysis of the metal content of soybean lipoxygenase-1 [*14*] is given in Table III.

Some non-transition metals have been shown to cause varying effects on the oxygenating activity of lipoxygenase. However, no pertinent data are available showing that these ions interact in a specific way with the protein. Verhagen *et al.* [*23*] have demonstrated that the stimulatory effect of Ca^{2+} ions observed for lipoxygenase-2 from soybeans most probably is due to the binding of substrate fatty acid by these ions, thereby lowering the actual fatty acid concentration. The very pronounced substrate inhibition exhibited by this type of enzyme is then partly alleviated. The effects of Ca^{2+} and Mn^{2+} ions on the lipoxygenase activity in chloroplasts have been described by Douillard and Bergeron [*24*]. The available evidence suggests that the activity of the enzyme(s) is primarily controlled by pH and only indirectly by cations like Ca^{2+} and Mn^{2+}. The various effects of calcium ions on lipoxygenase isoenzymes from peanuts have been described by Nelson *et al.* [*25*]. Ca^{2+} was found to have an inhibitory effect on peanut lipoxygenase-1 whereas it activated isoenzymes 2 and 3. Matsuda *et al.* [*26*] have reported the presence of protoheme IX in a protein from the fungus *Fusarium oxysporum* showing lipoxygenase activity. In addition, this enzyme requires Co^{2+} for optimal activity.

TABLE III Metal Analysis of Soybean Lipoxygenase-1[a]

Fe	Cu	Mn	Ca	Mg	Zn	Co	V	Mo	Ni
0.912	0.0018	0.086	0.21	0.18	0.03	—	—	—	—

[a] Mol metal/mol enzyme; MW 98,500.

VI. SUBSTRATES AND PRODUCTS

A. Substrates

Lipoxygenase is generally defined in terms of its capacity to oxygenate polyunsaturated fatty acids having a 1,4-*cis*,*cis*-pentadiene system. In a series of isomeric octadecadienoic acids, linoleic acid (all cis n-6, n-9 18:2) was found to be the best substrate for soybean lipoxygenase-1 by Holman *et al.* [*27*]. Only the all cis n-2, n-5 18:2 appeared to be converted at a reasonable rate, i.e., about 50% compared to linoleic acid. A reasonable rate is also observed for the following 18:3 acids: γ-linolenic acid (all cis n-6, n-9, n-12); and α-linolenic acid (all cis n-3, n-6, n-9). Only limited information is available on the effect of fatty acid chain length. The following C_{20} acids can also be used as substrates: bis(homo)-linoleic acid (all cis n-6, n-9 20:2); bis(homo)-γ-linolenic acid (all cis n-6, n-9, n-12 20:3); and arachidonic acid (all cis n-6, n-9, n-12, n-15 20:4). Much less is known about the substrate specificity of other plant lipoxygenases. The majority of these enzymes are considered to have a pH optimum lower than that for soybean lipoxygenase-1. At such pH values a dispersing agent is required in order to have suitable assay conditions. It has been reported that methyllinoleate [*28–30*], unfractionated seed oils [*29*], and mono- and dilinolein [*29*] are converted by some of these enzymes. This suggests that in these cases the presence of a carboxyl group is not a prerequisite.

Linoleyl sulfate [*2,32–34*] and several other linoleyl derivatives have been tested as substrates for soybean lipoxygenase isoenzymes [*34*]. Linoleyl sulfate appeared to be the best substrate for lipoxygenase-1 whereas lipoxygenase-2 and -3 were totally inactive toward this substrate. As of now, animal lipoxygenases are poorly characterized with respect to substrate specificity. For *in vitro* experiments, arachidonic and bis(homo)-γ-linolenic acids are commonly used as substrates.

B. Products

1. Primary Products

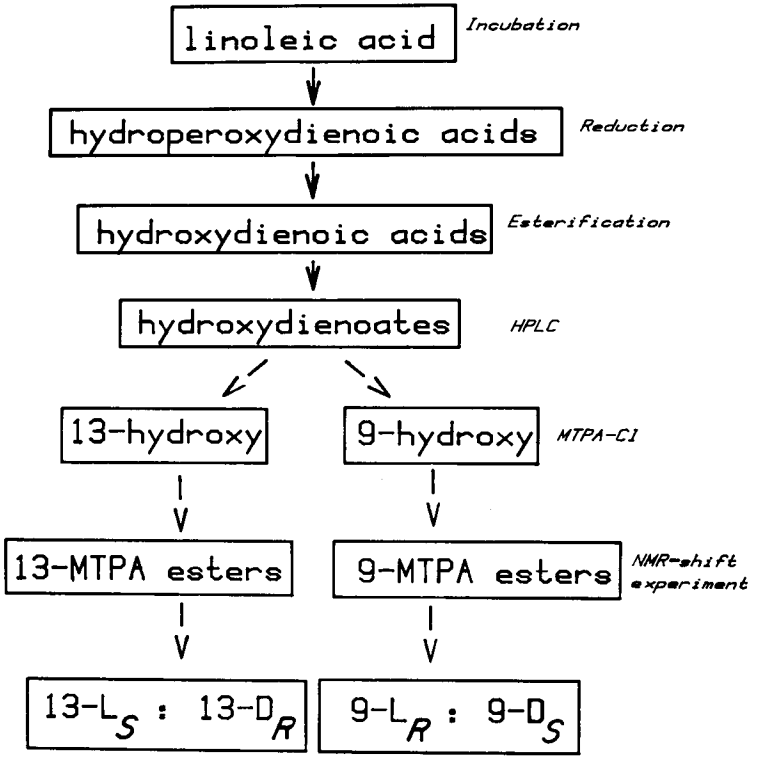
The primary products of the enzymic oxygenation of polyunsaturated fatty acids are cis,trans-conjugated monohydroperoxy fatty acids. For a characterization of this reaction, it is necessary to describe the positional isomers and stereospecificity of the product formation. The regiospecificity of this reaction depends on the enzyme (source and purity), the type of fatty acid, and the incubation conditions (concentrations of

the reactants and temperature). In general, a mixture of positional isomers will be obtained, e.g., with linoleic acid as the substrate, a mixture of 9- and 13-hydroperoxylinoleic acids is formed. The ratio of the amounts of the positional isomers can be established by mass spectrometry of the corresponding methylhydroxylinoleates, by thin layer chromatography (tlc) of ^{14}C-labeled hydroxylinoleates, or by high-performance liquid chromatography of either the hydroxy- or hydroperoxy fatty acids. Undoubtedly, the latter technique offers the method of choice since a good separation of the isomeric fatty acids can also be achieved in their underivatized form [*11*].

The regiospecificity as such has been applied as a criterion for the enzymic nature of the formation of hydroperoxides. However, this holds only for extreme ratios of the isomers becuase Chan *et al.* [*35,36*] have shown that the oxygenation of linoleic acid by transition metal ions and by metalloproteins proceeds with a definite regioselectivity. Hydroperoxide formation by lipoxygenase comprises two chiral steps: the removal of hydrogen from the methylene group of the 1,4-*cis,cis*-pentadiene system and oxygen insertion at either C-1 or C-5 of the pentadiene system. Egmond *et al.* [*37*] have concluded that with preparations of soybean lipoxygenase-1 and corn germ lipoxygenase an antarafacial relationship exists between hydrogen abstraction and oxygen insertion. Hamberg and Hamberg [*38*] have demonstrated that the process of hydroperoxide formation from arachidonic acid by a lipoxygenase from blood platelets has identical stereochemical features. Generally, only the chirality of the products can be employed to decide on involvement of an enzyme as catalyst.

Several methods can be applied to determine the enantiomeric composition of oxygenated fatty acid derivatives. Optical rotation measurements require extensive purifications because of the low specific rotations of hydroxy and hydroperoxy fatty acids (cf. [*39*]). Hamberg [*40*] has developed a gas chromatographic method for the determination of the enantiomeric composition of the hydroperoxy octadecadienoic acids formed from linoleic acid. This approach was based on the gas chromatographic separation of diastereomeric menthyloxyformate esters derived from the hydroxy octadecadienoates. Van Os *et al.* [*41*] introduced a convenient gas chromatographic procedure in which ozonolysis of the acetylated hydroxy dienoates yields acetylated 2-hydroxy acids. The latter are converted into *R*(−)-2-butyl esters and then reacetylated. The enantiomeric composition of these preparations can be determined by capillary gas chromatography [*41*]. According to an earlier suggestion [*2*], Van Os *et al.* [*42*] demonstrated that after separation of the positional isomers, the enantiomeric composition can be derived from an nmr-shift

experiment. The positional isomers of methyl esters of the hydroxy fatty acids are separated by high pressure liquid chromatography (HPLC) and then subjected to a chiral derivatization to form diastereomeric α-methoxy-α-trifluoromethylphenyl acetate esters. A determination of the diastereomeric composition can be performed with lanthanide shift reagents like $Eu(fod)_3$ and $Pr(fod)_3$ (Fig. 2, Scheme 2).



Scheme 2. Flow chart of the procedure in the determination of the regio- and stereoselectivity of lipoxygenase reactions.

The method seems to be generally applicable for all common chiral hydroxy fatty acids containing from one to four double bonds. Recent data on the product specificities of lipoxygenases from different sources are summarized in Table IV.

It is noteworthy that for each enzyme a set of conditions can be found leading to the formation of mainly chiral products. For soybean lipoxygenase-2 and for lipoxygenase from peas, generally a pH optimum of 6–7 is presumed. However, at pH 9.0 and at low substrate concentra-

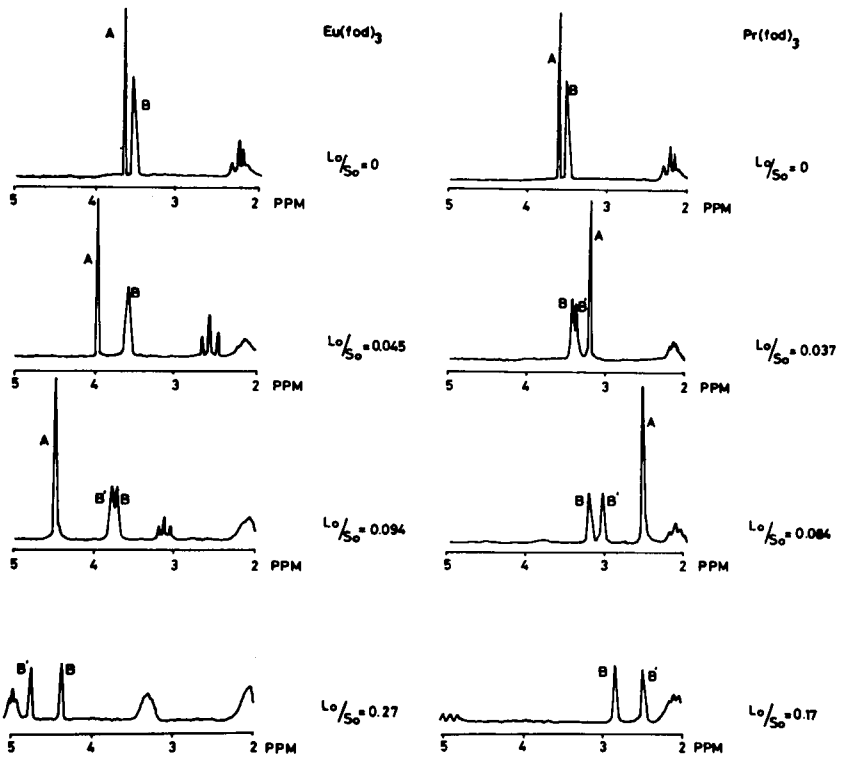


Fig. 2. Lanthanide shift experiments with diastereomeric mixtures of MTPA esters derived from racemic hydroxylinoleate. L_o, concentration of lanthanide shift reagent; S_o, concentration of MTPA ester; A, methoxy signal from the lipid; B′, methoxy signal from the MTPA moiety of the diastereomer derived from *S*-hydroxydienoate (13-L or 9-D); B, methoxy signal from the MTPA moiety of the diastereomer derived from *R*-hydroxydienoate.

tions, specifically 9-L(*R*)-hydroperoxy-10-*trans*-12-*cis*-octadecadienoic acid is produced by these enzymes. At higher substrate concentrations the enzyme is inhibited. Since the degree of inhibition depends on the enzyme concentration, it cannot be considered a normal type of substrate inhibition but rather may be related to a kinetic lag phase, a well-known phenomenon in lipoxygenase reactions (see Section VIII). The capability of lipoxygenase-2 to produce the 9-L(*R*)-hydroperoxide may have some relevance for the explanation of the formation of immunologically active prostaglandins from arachidonic acid [*56*]. Hamberg and Samuelsson reported [*51*] that fatty acid cyclooxygenase forms specifically the 9-L(*R*)-hydroperoxide from linoleic acid, whereas Hamberg and Hamberg [*38*] found that from arachidonic acid specifically the 12-L(*S*) enantiomer

TABLE IV Stereospecificities of Various Lipoxygenases[a]

Enzyme	Substrate	Conditions	Regiospecificity	Stereospecificity	Reference
Plant systems					
Soybean-1	LH	pH 9.0	13:9 = 96:4	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 93.1:2.9:1.6:2.4	*40*
Soybean-1	LH	pH 6.6	13:9 = 77:23	13-L(*S*);13-D(*R*):9-L(*R*):9-D(*S*) = 63:5:2:21	*42*
Corn germ	LH	pH 6.5	13:9 = 7:93	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 3.5:3.5:4.0:89	*40*
Soybean-2	LH	pH 6.6	13:9 = 25:75	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 12.5:12.5:41:34	*43*
Soybean-2	LH	pH 9.0	13:9 = 35:65	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 30:5:51:14	*43*
Pea	LH	pH 9.0	13:9 = 41:59	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 37:4:37:22	*43*
Pea	LH	pH 6.6	13:9 = 33:67	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 17:17:33:33	*43*
Thea sinensis chloroplasts	LH	—	13:9 = 84:16	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 67:17:6.8:9.3	*44*
Dimorphotica sinuata	LH	pH 6.9	100% 13	mainly 13-L(*S*)	*45*
Peanut	LH	pH 6.2	13:9 = 86:14	mainly 13-L(*S*) and 9-D(*S*)	*46*
Potato	LH	pH 5.5	13:9 = 5:95	mainly 9-D(*S*)	*47*
Tomato	LH	pH 5.5	13:9 = 4:96	mainly 9-D(*S*)	*48*
Soybean-1	all cis(n-2), (n-5)18:2	pH 9.0	17:13 = 85:15	mainly 17-L(*S*)	*49*
Soybean-1	ARAH	pH 9.0	100% 15	mainly 15-L(*S*)	*50*
Animal systems					
Cyclooxygenase	LH	pH 7.4	13:9 = 23:77	13-L(*S*):13-D(*R*):9-L(*R*):9-D(*S*) = 18:5:61:16	*51*
Platelets	all cis(n-6), (n-9), (n-12)20:3	pH 7.0	100% 12	12-L(*S*):12-D(*R*) = 91:9	*52*
Human platelets	ARAH	pH 7.0	100% 12	mainly 12-L(*S*)	*53*
Guinea pig lung	ARAH	pH 7.4	100% 12	mainly 12-L(*S*)	*54*
Rabbit PMNL	ARAH	KRB	100% 5	5-L(*R*):5-D(*S*) = 17:83	*55*
Rabbit PMNL	all cis(n-6), (n-9), (n-12)20:3	KRB	100% 8	8-L(*R*):8-D(*S*) = 18:82	*55*

[a] Abbreviations: LH, linoleic acid; ARAH, arachidonic acid; PMNL, polymorphonuclear leucocytes; KRB, Krebs–Ringer buffer.

is produced. In both cases an antarafacial relationship between hydrogen abstraction and oxygen insertion was established.

2. Secondary Products

Lipoxygenase not only forms hydroperoxides from unsaturated fatty acids but also varying amounts of secondary products. A prominent representative of these secondary products is the hydroxy analog. Such a hydroxy fatty acid may be formed by reduction either by the enzyme itself or by contaminating or associated proteins, e.g., the peroxidase that is present in the prostaglandin synthetase complex [*51*]. Disproportionation of the hydroperoxide can also be the origin of hydroxy acids. This possibility is more likely in cases where oxodienoic acids are also found. Relatively low oxygen concentrations may give rise to products which are typical for the anaerobic reaction of soybean lipoxygenase-1. Pattee *et al.* [*57,58*] have reported the formation of pentane and hexanal from linoleic acid even under aerobic conditions; however, the remaining part of the molecule was not identified. Pentane and other hydrocarbons can also be produced nonenzymically from unsaturated fatty acid hydroperoxides [*59,60*].

Product hydroperoxides formed from unsaturated fatty acids with more than two methylene interrupted double bonds can, under favorable reaction conditions, act as a substrate for lipoxygenase (see also Section VIII,D). With arachidonic acid as the primary substrate at pH 10, the first dioxygenation step leads to the formation of exclusively 15-L(*S*)-hydroperoxy-5-*cis*,8-*cis*,11-*cis*,13-*trans*-eicosatetraenoic acid [*50,61*]. In the second step at pH 8.5, two isomeric dihydroperoxy fatty acids are formed namely 8-D(*S*),15-L(*S*)-dihydroperoxy-5-*cis*,9-*trans*,11-*cis*,13-*trans*-eicosatetraenoic acid and 5-D(*S*),15-L(*S*)-dihydroperoxy-6-*trans*,8-*cis*,11-*cis*,13-*trans*-eicosatetraenoic acid in a ratio of 3:2. On the basis of the results described above, the conclusion can be drawn that soybean lipoxygenase-1 produces hydroperoxides having mainly the *S* configuration irrespective of the position in the fatty acid that is oxygenated.

VII. SPECTROSCOPIC STUDIES

Early indications for the interaction between lipoxygenase and fatty acid hydroperoxides came from Haining and Axelrod [*62*] who described a kinetic lag phase in the formation of hydroperoxides from linoleic acid. They also noted that the duration of such a lag phase could be shortened by the addition of extra amounts of product hydroperoxide.

Following the discovery of the anaerobic reaction by Garssen *et al.* [*5,10*] and kinetic studies by Smith and Lands [*15*], direct evidence for a specific interaction between lipoxygenase-1 and hydroperoxylinoleic acid was obtained by Finazzi-Agrò *et al.* from fluorescence spectroscopy [*13*]. It was found that the protein fluorescence was quenched by adding product hydroperoxide. This quenching effect was found to be directly dependent on the amount of hydroperoxide added with the maximum effect occurring at a protein/product molar ratio of 1:1. The position of the fluorescence peak at 328 nm suggests that either the tryptophan residues of the protein are all in a nonpolar environment or differently solvated tryptophans have a much lower quantum yield.

Once lipoxygenase-1 could be obtained in larger amounts and in a purer state (see Section III; see also [*13*]) the application of other spectroscopic techniques became feasible. In particular, electron paramagnetic resonance (epr) and circular dichroism (cd) spectroscopy have greatly contributed to our knowledge of the protein and the role of iron in the catalytic mechanism. De Groot *et al.* [*63*] and Pistorius *et al.* [*64,65*] used epr spectroscopy to establish the functional role of iron. De Groot *et al.* [*63*] have proposed a mechanism for both the aerobic and anaerobic lipoxygenase catalysis in which a lipoxygenase species containing Fe(III) plays a key role. Furthermore, absorption and fluorescence spectroscopy [*66,68*] have furnished evidence for the existence of at least three distinct enzyme species: the native form [presumably containing Fe(II)], the yellow Fe(III) species and a purple Fe(III) species that is probably a complex of the yellow species, and the hydroperoxide. The absorption spectra of the various lipoxygenase species are given in Fig. 3. The molar absorption of the yellow enzyme at 330 nm is 1800 $M^{-1}\cdot cm^{-1}$, whereas the purple species has molar absorptions of 1960 and 1320 $M^{-1}\cdot$ cm^{-1} at 360 and 578 nm, respectively [*69*].

The kinetics of the interaction between hydroperoxylinoleic acid and soybean lipoxygenase-1 have been studied by Egmond *et al.* [*70*] and by Aoshima *et al.* [*71,72*] using stopped-flow techniques. The chemical conversion leading to the formation of the chromophore at 330 nm and the fluorescence quenching at 328 nm is probably as shown in Scheme 3 [*70*].

$$13\text{—L—LOOH} + \text{E—[Fe(II)]} \longrightarrow 13\text{—LO}\cdot + \text{E—[Fe(III)]} + OH^-$$

Scheme 3. Oxidation of lipoxygenase-1 by 13-L-LOOH.

Lipoxygenase from potato tubers, known to produce specifically 9-D(*S*)-hydroperoxylinoleic acid, has very similar fluorescence properties: the

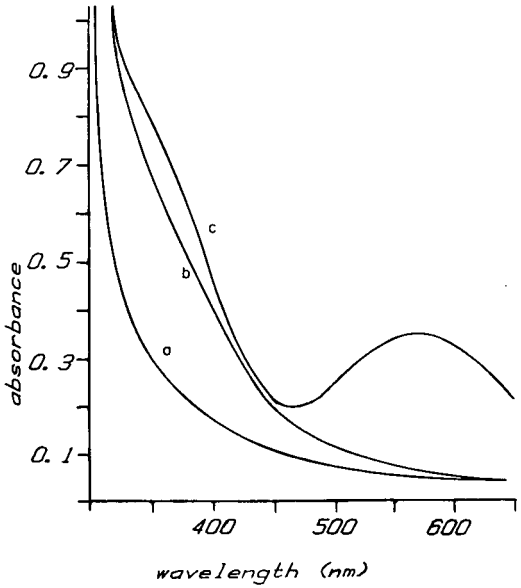


Fig. 3. Absorption spectra of soybean lipoxygenase-1: a, native enzyme; b, yellow enzyme; c, purple enzyme.

maximum emission at 328 nm is quenched by adding the 9-D(*S*)-hydroperoxide. Interestingly, the kinetic lag phase observed with this enzyme can also be reduced by adding the 9-D(*S*)-hydroperoxide [*73*].

The visible range of the cd spectrum of the native enzyme shows a negative dichroic band at 330 nm. The spectrum of the yellow enzyme has a positive Cotton effect at 425 nm while the negative band at 330 nm has increased in intensity. The purple enzyme species has a negative cd band at 580 nm and positive bands at 410 and 391 nm. The near ultraviolet cd spectra of the native, yellow, and purple enzyme species are very similar [*69*]. Table V contains the relative amounts of α-helix, β-form, and random coil of native and yellow lipoxygenase-1 as calculated from this part of the spectrum.

From 270 MHz ^{1}H-nmr studies on the native, yellow, and purple enzyme species, it was also concluded that a relatively large portion of

TABLE V Secondary Structure Parameters for Lipoxygenase-1

	α-Helix	β-Form	Random coil
Native	34.1	26.9	39.0
Yellow	31.5	24.9	43.6

the lipoxygenase-1 molecule was effectively random coil and that differences in the secondary structures between the various enzyme species were hardly discernable [*74*]. The purple species is not very stable at room temperature. On standing, it gradually turns into a fully active yellow species thereby converting the hydroperoxide into an epoxyhydroxymonoenoic acid [*75*]. Experiments with 4-nitrocatechol [*14,76*] have shown that this active-site probe and 13-hydroperoxylinoleic acid compete for the same site on the enzyme which can be considered as additional evidence for the existence of an enzyme–product complex (Fig. 4).

In subsequent epr spectroscopic investigations of soybean lipoxygenase-1 [*77*], the zero-field splitting constants (*D*) of the various components of the complicated high-spin Fe(III) spectrum were determined (Fig. 5). For a quantitative assessment of the amounts of epr-visible iron, *D* values should be known as accurately as possible. Two methods were used to determine the *D* values: (1) from the temperature dependence of the Fe(III) signals and (2) from the microwave frequency dependence. Inherent difficulties in the measurement of the temperature in a helium

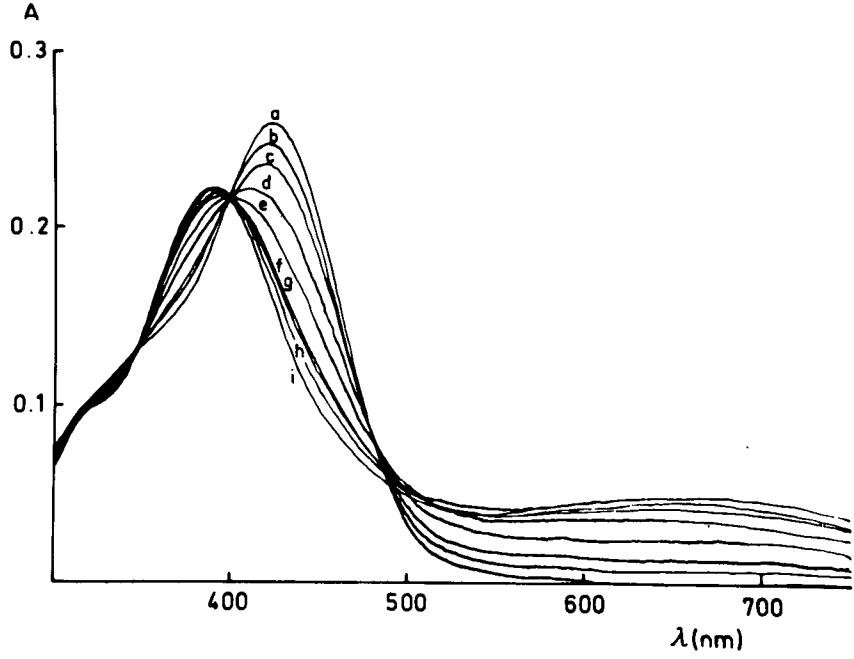


Fig. 4. Stepwise addition of Fe(III)–lipoxygenase-1 to 4-nitrocatechol (0.1 *M* sodium phosphate, pH 7.0, 20°C).

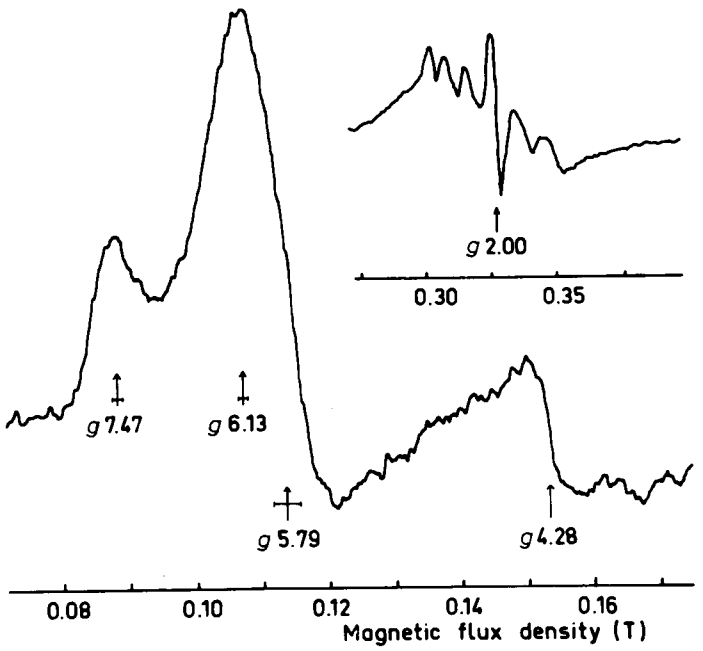


Fig. 5. epr Spectrum of yellow soybean Fe(III)-lipoxygenase-1 at 9.195 GHz. Insert: high field part of the spectrum.

flow system could be overcome by using the known temperature dependence of cytochrome *c* that was added to the lipoxygenase sample. For the axial and rhombic species the following ranges for *D* could be established: 1.5–3.0°K and 1.8–4.4°K, respectively. In contrast to another nonheme iron dioxygenase (see [*78,79*]; cf. [*64*]), *D* values of iron in lipoxygenase thus appear to be positive. Slappendel *et al.* [*80*] determined the actual amount of iron that contributes to the epr signals by simulating the spectra. Table VI summarizes the data on the epr-visible iron.

TABLE VI epr-Visible Iron of Soybean Lipoxygenase-1[a,b]

Enzyme species	*g* 6	*g* 4.3
Native	<1	0.1
Yellow	75	0.5
Purple	76	11

[a] From Slappendel *et al.* [*80*].
[b] Percent of total amount of iron.

epr Spectra of complexes between soybean lipoxygenase and nitrogen oxide (NO) have also been described [*81,82*]. The NO complex of the native enzyme shows Fe resonances near g 4 and below g 2 which have not been observed previously in a well-defined protein entity. Lipoxygenase may therefore represent a novel class of nonheme iron centers. The only model system showing similar resonance lines is Fe(II)–EDTA–NO [*83*]. Also, the properties of some other Fe–EDTA complexes may be relevant as a model system for lipoxygenases [*84,85*].

Only few lipoxygenases from sources other than soybeans have been studied by epr spectroscopy. Spaapen *et al.* [*86*] have described the epr properties of pea lipoxygenase which appear to be similar to those of soybean lipoxygenase. A lipoxygenase-type of enzyme from fungal origin [*87*] containing protoheme IX as prosthetic group and requiring Co(II) as a stabilizing factor has been studied by epr and cd spectroscopy [*88*]. The presence of a different prosthetic group will most probably account for the differences between the spectroscopic properties of this type of enzyme and those of the nonheme enzymes from soybeans and peas.

VIII. KINETICS

A. Aerobic Reaction

On many occasions a systematic investigation of the kinetics of an enzymic reaction has significantly contributed to the understanding of the mechanism of the reaction. This also applies to lipoxygenase although a number of points still need to be clarified. The net aerobic reaction of lipoxygenase is summarized below (Scheme 4).

$$LH + O_2 \longrightarrow LOOH$$

Scheme 4. LH, linoleic acid; LOOH, hydroperoxylinoleic acid.

We will largely confine ourselves to a discussion of results obtained with lipoxygenase-1 from soybeans because only very few data are available on other enzymes. The enzyme can exist in different forms with iron either epr-silent and presumably in a ferrous state, or in a ferric state. The substrate fatty acid has very limited solubility in aqueous solutions. Upon increasing the substrate concentration beyond a certain threshold level, the monomeric substrate molecules start to aggregate and eventually form micelles which may or may not have an influence on the rate of the reaction. Dissolved molecular oxygen is the second substrate in the aerobic reaction. Also, this substrate has a limited sol-

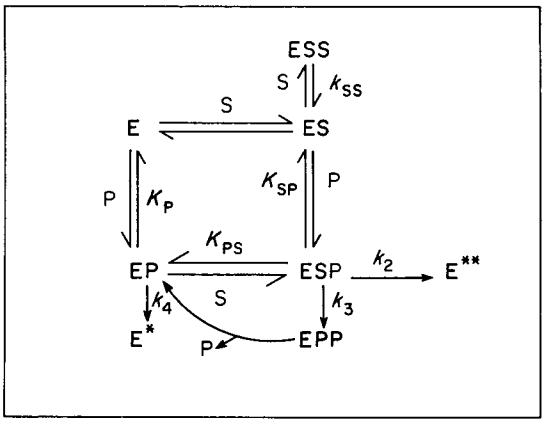
ubility in aqueous buffers which is, however, comparable to linoleic acid. Furthermore, the product hydroperoxide is known to affect the kinetic behavior of soybean lipoxygenase-1. For example, the hydroperoxide is capable of converting the native enzyme into a yellow species thereby oxidizing the iron into a ferric state. In addition, the hydroperoxide can form a purple-colored enzyme–product complex which may subsequently revert to a yellow active form or to an inactive enzyme species. With so many factors involved, a comprehensive study of the kinetics of the aerobic lipoxygenase reaction requires an extensive experimental and theoretical effort. In spite of these facts quite a number of studies have appeared aimed at elucidating the catalytic mechanism by studying the kinetics of the lipoxygenase reaction. For earlier studies the reader is referred to review articles that include a discussion of kinetic aspects of lipoxygenase reactions [*2,89*]. Smith and Lands [*15*] have proposed a mechanism in which an activation of the native enzyme by product hydroperoxide is a compulsory step. This study was carried out with arachidonic acid as the primary substrate. The activating role of the hydroperoxide was evident from its effect on the kinetic lag phase. On one hand this lag phase can be abolished completely by adding product hydroperoxide to the assay mixture, while on the other hand the lag can be extended in such a way that virtually no reaction takes place by adding the enzyme glutathione peroxidase (EC 1.11.1.9; GSH:H_2O_2 oxidoreductase) which is known to react readily with fatty acid hydroperoxides [*7,8*] in the presence of glutathione. In a subsequent study [*90*], some of the kinetic parameters were refined and interesting results were obtained by comparing the experimental results with those predicted from computer analyses of kinetic models. The results of these studies are summarized in Table VII and Scheme 5.

Lagocki *et al.* [*91*] have studied the kinetics of lipoxygenase reacting

TABLE VII Kinetic Parameters of the Oxygenation of Arachidonic Acid by Soybean Lipoxygenase

k_3	24000	μ*M*/mg/min
k_2	0.51(±)0.1	/min
k_4	0.06(±)0.02	/min
K_{ps}	1.0	μ*M*
K_{sp}	0.6	μ*M*
K_p	1.0	μ*M*
K_s	1.7	μ*M*
K_{ss}	30	μ*M*

[a] From Cook and Lands [*90*].



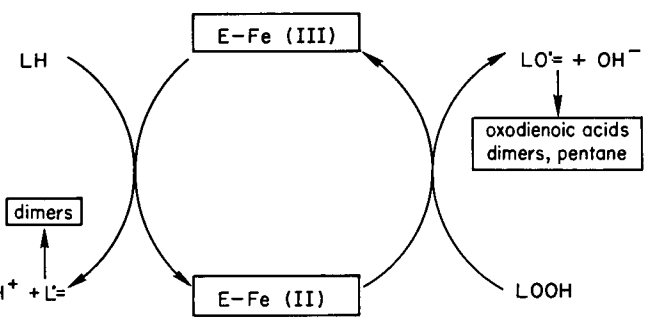
Scheme 5. Kinetic scheme for lipoxygenase action. (From Cook and Lands [*90*].)

with linoleic acid. They have proposed a model in which the product can also bind to the substrate site. The kinetic constants were derived from an analysis of the full time course of the oxygenation reaction as measured by following the change in the absorbance at 234 nm with time. A dissociation constant of the EPS–complex (K_{ps}) of 7.7 μM was found. Spaapen *et al.* [*21*] reported a similarly low K_m value for linoleic acid after taking into account the possible influence of micelle formation [*91–93*] and by including velocity measurements at relatively low linoleic acid concentrations (cf. [*34,94–97*]). Lagocki *et al.* [*91*] also recognized the possible importance of micelle formation and found, under the conditions of their routine lipoxygenase assay, a critical micelle concentration of 150 μM. This kinetic analysis did not substantiate the activating role of the hydroperoxy group of the product but instead this effect was ascribed to the 1,3-diene system of the product. This conclusion was drawn after studying the effect of adding sodium borohydride ($NaBH_4$) to an assay mixture, which conceivably, could have an effect similar to the addition of glutathione peroxidase/GSH (cf. [*90*]). In the study of Lagocki *et al.*, $NaBH_4$ was found to have no effect on the reaction rate. These authors also reported that reaction rates were independent of the initial state of the enzyme, i.e., whether or not it had been pre-incubated with product hydroperoxide. Contrary to these observations, Gibian and Galaway [*94*] have established an activating role for the hydroperoxy group of the product but confirmed the inhibitory effect of the product also reported by Lagocki *et al.* [*91*]. Gibian and Galaway [*94*] proposed a chemical activation of the native enzyme by the product hydroperoxide

which was also suggested by De Groot *et al.* [*63*] on the basis of epr-spectroscopic data. However, any intrinsic differences between the native enzyme and the product-treated enzyme did not emerge from apparently straightforward comparative kinetic experiments [*21,91*] in which both enzyme forms were incubated with substrate fatty acids.

B. Anaerobic Reaction

A mechanism for the anaerobic catalytic activity of soybean lipoxygenase-1 has been proposed on the basis of an investigation of the steady-state kinetics by Verhagen *et al.* [*11*]. As substrates, linoleic acid (LH) and 13-L-hydroperoxylinoleic acid (LOOH) were used. LH and LOOH are thought to react with lipoxygenase species containing iron in a ferric and ferrous state, respectively. This cycle is depicted in Scheme 6.



Scheme 6. LH, linoleic acid; LOOH, 13-L-hydroperoxylinoleic acid.

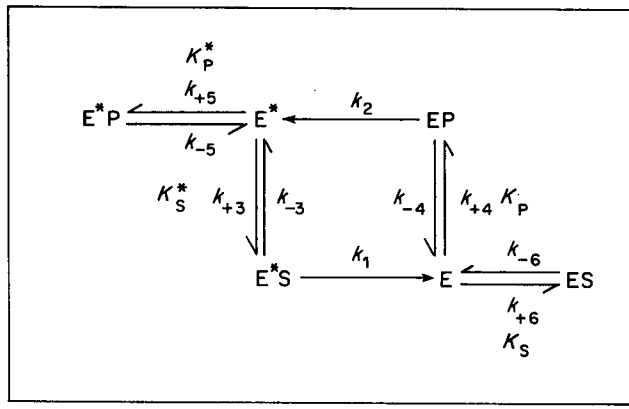
The occurrence of fatty acid free radicals has been demonstrated by using a spin-trapping technique [*98*], which has also been used to detect free radicals in the aerobic reaction of potato lipoxygenase [*73*].

The left half-cycle of the scheme represents the reduction of the ferric species by linoleic acid while the right part summarizes the subsequent oxidation of the ferrous enzyme by the fatty acid hydroperoxide. In fact, both these half-reactions have been studied separately by Egmond *et al.* [*70*] using a stopped-flow technique. This already imposed an important restriction on the number of possible mechanisms that had to be considered: any mechanism involving a compulsory ternary complex of linoleic acid, hydroperoxylinoleic acid, and enzyme could be excluded. The proposed substituted-enzyme ("ping-pong") mechanism was derived from fitting initial velocities to various theoretical models. Table VIII lists the values for the kinetic parameters and Scheme 7 is the corresponding kinetic model.

TABLE VIII Kinetic Parameters of the Anaerobic Reaction of Soybean Lipoxygenase-1 with Linoleic Acid and 13-L-Hydroperoxylinoleic Acid[a,b]

Parameter	Value		S.E.[c]
K^*_{ms}	55	±	6 μM
K_{pm}	73	±	8 μM
K_s	111	±	14 μM
K^*_p	153	±	25 μM
V	143	±	11 μM/min
K^*_s	163	±	22 μM
K_p	110	±	14 μM
K_1	309	±	10/s
K_2	156	±	16/s

[a] From Verhagen *et al.* [*11*].
[b] At pH 10.0.
[c] The experimental standard error (S.E.) was found to be 8%.



Scheme 7. E*, E-Fe(III); E, EFe(II); S, linoleic acid; P, 13-L-hydroperoxylinoleic acid.

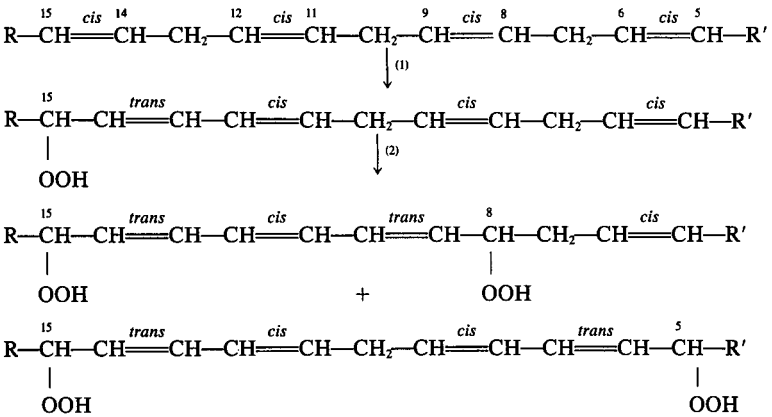
It was found that linoleic acid and hydroperoxylinoleic acid were converted in this reaction in an exact 1:1 molar ratio. Invariably, the amount of oxodiene formed from these substrates is 50% of the substrate that ultimately limits the extent of the reaction.

An examination of the deuterium isotope effect in the anaerobic reaction by Verhagen [*99*] has provided additional support for this mechanism. Initial rates of mixtures of 11,11-dideuterolinoleic acid and 13-L-hydroperoxylinoleic acid were measured and compared with those predicted from the known kinetic parameters and a deuterium isotope effect of 8.7 in the aerobic reaction as determined by Egmond *et al.* [*95*]. The

deuterium isotope effect was found to depend on the ratio of the two substrates in a way that is to be expected from the proposed model. The experimental and theoretical data were found to be in close agreement.

C. Double Dioxygenation of Arachidonic Acid

Bild *et al.* [*100,101*] have reported the capacity of soybean lipoxygenase-1 to incorporate two dioxygen molecules in fatty acids containing a 1,4,7-octatriene system like arachidonic acid. With arachidonic acid as the substrate, the reaction product was identified by these authors as 8,15-dihydroperoxy-5,9,11,13-eicosatetraenoic acid. In a subsequent study, Van Os *et al.* [*61*] reported the formation of the 8,15- and the 5,15-dihydroperoxy compounds in a 3:2 ratio. The shape of the progress curve of the oxygen consumption already suggested that the reaction occurs in two consecutive steps: in the first step the 15-hydroperoxy arachidonic acid is formed while in a second step the hydroperoxide is the true substrate for lipoxygenase which is converted into the dihydroperoxy derivatives (Scheme 8).



Scheme 8. Dioxygenation of arachidonic acid and 15-hydroperoxyarachidonic acid by soybean lipoxygenase-1.

In addition to arachidonic acid Bild *et al.* [*101*] also examined the double dioxygenation of a number of other polyunsaturated fatty acids. From these studies it has become clear that any fatty acid containing

TABLE IX Kinetic Parameters of the Double Dioxygenation of Arachidonic Acid

	Step 1	Step 2	Ref.
K_m	8.5 μM,pH 10	440 ± 20 μM,pH 8.7	*61*
K_m	86 μM,pH 9	18 μM,pH 7.5	*101*
K_{ps}	1.0 μM,pH 8.5	—	*90*

a *cis,cis,cis*-1,4,7-octatriene system will undergo a second dioxygenation step provided that after the first step a *cis,cis*-1,4-pentadiene system remains. The kinetics and the pH dependence of the two dioxygenation processes differ significantly, the second step being slower by several orders of magnitude. Kinetic parameters reported by Bild *et al.* [*101*], Van Os *et al.* [*61*], and by Cook and Lands [*90*] are compiled in Table IX.

D. Oxygenation of Linoleic Acid by Modified Lipoxygenase-1

A number of small organic mercurials have been used by Spaapen *et al.* [*21*] to study the effects of modifying sulfhydryl groups in lipoxygenase-1 (see also Section X). In particular, some of the kinetic properties of lipoxygenase-1, modified by methylmercuric halides, had changed. Compared to the nonmodified enzyme the length of the kinetic lag phase had increased. It was found that over a wide range of linoleic acid concentrations, a steady state in the rate of oxygen consumption was reached only after 20–25% of the substrate had been converted. For the native enzyme this amounts to 6%. The extension of the lag phase leads to a kinetic pattern that can easily be misinterpreted as a normal type of substrate inhibition. However, because the degree of inhibition depends on the enzyme concentration, the latter can be chosen such that the initial parts of the progress curves are linear with time and a normal Michaelis–Menten behavior is observed. Compared with the native enzyme there is no apparent change in the Michaelis–Menten constant (3.3 μM, substrate: linoleic acid; pH 10, 0.1 *M* sodium borate). Meanwhile, the catalytic constant has changed from 157 (native enzyme) to 79/s for the modified species. After treatment of the modified enzyme with NaHS all the original catalytic properties of the native enzyme could be restored [*21*].

IX. INHIBITORS

The enzymic oxygenation of linoleic acid by lipoxygenase can be inhibited by various antioxidants as has been demonstrated by Holman

[*102*], Tappel *et al.* [*103,104*], and Yasumoto *et al.* [*105*]. For example, nordihydroguaiaretic acid (NDGA) competitively inhibits the oxygenation reaction. The effect of these inhibitors was ascribed to the antioxidant properties of these agents because at that time no metal cofactor was known for lipoxygenase. Since iron has been found in the enzyme, it cannot be excluded that these compounds exert an effect by coordinating to iron.

4-Nitrocatechol and some other *O*-diphenolic compounds like protocatechualdehyde, 2,3-dihydroxyanthraquinone (histazine), catechol, 7,8-dihydroxy-4-coumarin, and 3,4-dihydroxybenzonitrile inhibit the yellow enzyme form of soybean lipoxygenase-1 by interacting with its Fe(III) as demonstrated by Galpin *et al.* [*14*]. A detailed study of the properties of the complex of 4-nitrocatechol and soybean Fe(III)-lipoxygenase-1 by Spaapen *et al.* [*20,76*] has shown that a green 1:1 complex is formed having absorption maxima at 385 and 650 nm at pH 7.0. On the basis of steady-state kinetics it was concluded that the inhibition of Fe(III)-lipoxygenase-1 by 4-nitrocatechol is of a noncompetitive type. However, because a state of equilibrium is reached very slowly the inhibition may rather be pseudo noncompetitive [*76*]. Prolonged incubation of the complex leads to conversion of the green complex into a brown one. Concomitantly, the type of inhibition changes from reversible to irreversible. This observation might explain the apparent irreversibility of the inhibition by 4-nitrocatechol reported by Galpin *et al.* [*14*]. The inhibition of lipoxygenase-1 by iron-chelating agents has been described by Chan [*106*], Roza and Francke [*107*], and Pistorius and Axelrod [*65*]. According to Naim *et al.* [*108*] soybean lipoxygenase-1 can be inhibited by isoflavones from soybeans. Experiments described by Fiebrich and Koch [*109*] suggest an inhibitory effect of silymarin. Palla and Verrier [*110*] have described the inhibition by certain hydroquinone derivatives. Mitsuda *et al.* [*17*] discovered that hydrogen peroxide is a very efficient, irreversible inhibitor of soybean lipoxygenase-1. This observation has been confirmed by Egmond *et al.* ([*66*]; cf. [*2*]). A chemical reaction between H_2O_2 and the enzyme can be held responsible for this phenomenon: namely, the conversion of the native enzyme into a yellow-colored Fe(III) species. In this process hydroxyl radicals probably are formed giving rise to structural alterations in the coordination sphere of iron. The fluorescence, absorption [*66*], and epr spectra of the enzyme treated with a four-fold molar excess of hydrogen peroxide [*111*] show a great resemblance with those of the active yellow Fe(III) species. Spaapen [*20*] could show that the cd spectra of soybean lipoxygenase-1 samples treated with an equimolar amount of hydrogen peroxide and 13-hydroperoxylinoleic acid are significantly different. After hydrogen peroxide treatment, the spectra show positive dichroic bands at around 325 and 410

nm, whereas in the latter case a negative cd band at 340 nm and a positive one at 425 nm was observed. Further addition of hydrogen peroxide up to a sixfold molar excess leads to a strong decrease of the cd band at 410 nm but the near ultraviolet cd spectrum remains unchanged. This suggests that only the iron chromophore is susceptible to attack by hydrogen peroxide. This is in line with the observation of Mitsuda *et al.* [*17*] that certain substrate analogs like linoelaidic acid or conjugated linoleic acid are capable of protecting the enzyme against attack by hydrogen peroxide. Aoshima *et al.* [*112*] concluded on the basis of a stopped-flow kinetic study that the interaction between lipoxygenase and hydrogen peroxide can be conceived as a simple irreversible one-step mechanism. Interestingly, they found that 13-hydroperoxylinoleic acid protected the enzyme against inactivation by hydrogen peroxide. They suggested that amino acids essential for the catalytic activity of the enzyme are modified by free radicals, e.g., hydroxyl radicals. Another type of compound known to have inhibitory effects in lipoxygenase reactions are fatty acids containing triple bonds [*113–115*]. These compounds affect enzymes from plant as well as from animal origin. Experimental results with this class of compounds [*99*] cast some doubt on the hypothesis that the mechanism of action of these inhibitors involves hydrogen abstraction from the (n-8) carbon atom leading to the formation of an allenic compound that may then react irreversibly with the protein.

Since only a few reports are available on the inhibition of the enzyme through amino acid modifications, only little is known about the amino acid residues in the active site of lipoxygenase-1. Mitsuda *et al.* [*17*] ascribe the inactivation by hydrogen peroxide to the destruction of a histidine residue. The effects of hydrogen peroxide and of photooxidation as studied by Aoshima *et al.* [*112*] suggest that a tyrosine or an abnormal histidine residue is modified. *N*-Bromosuccinimide can also bring about a considerable inactivation, e.g., with lipoxygenases from rice bran [*116*] and potatoes [*117*] which may point to essential tryptophan residues. Spaapen *et al.* [*21*] have demonstrated that modification of thiol groups with organic mercurials leads to an enzyme with a greatly decreased catalytic activity.

X. MODIFIED LIPOXYGENASE-1

Spaapen *et al.* [*21*] have carried out a comparative study of native soybean lipoxygenase-1 and an enzyme species obtained by chemically modifying lipoxygenase-1 with a methylmercuric halide. Without the

presence of a denaturing agent, none of the sulfhydryl groups was accessible to sulfhydryl reagents (see Section IV). By including appropriate amounts of either sodium dodecylsulfate or guanidine HCl, five sulfhydryl groups could be determined. From incubations with ^{14}C-labeled methylmercuric iodide it was concluded that 3.3 mol of this reagent had reacted per mol of protein. Such a modified lipoxygenase-1 was subsequently found to contain 1.5 free sulfhydryl groups. Table X summarizes the numbers of free sulfhydryl groups per mol of enzyme and the corresponding oxygenating capacities.

As can be seen from Table X, the addition of NaHS completely restores the activity of the enzyme as well as the number of free sulfhydryl groups. The reactivation could not be achieved with various other compounds, e.g., β-mercaptoethanol, dithioerythritol, and cysteine. The modification affects a number of other catalytic properties of lipoxygenase-1. The kinetic lag phase was found to be considerably longer than for the native protein. Also, the effect of the product hydroperoxide (13-L-LOOH) on the length of the lag phase had changed. A steady state was reached only after 20–25% of the substrate had been converted, whereas with the native enzyme a 6% conversion is sufficient (see Section VIII for other kinetic differences between the native and the modified enzyme).

Lipoxygenase isoenzymes differ in their capacities to co-oxidize suitable co-substrates like canthaxanthine, β-carotene, and crocin [*118*]. The type-2 enzymes are superior to the type-1 enzymes in catalyzing co-oxidation reactions. If an enzyme species with a relatively large co-oxidizing capacity finds none of the above mentioned co-substrates, it is likely to treat part of the normal fatty acid substrate as a co-substrate [2]. Therefore, the fatty acid is then oxygenated in a less stereospecific way. The methylmercury-modified lipoxygenase-1 was found to have an enhanced co-oxidizing capacity as compared to the nonmodified enzyme (Table XI) [*21*].

The partial loss of enzymic control of the stereo- and regioselectivities

TABLE X Sulfhydryl Content and Oxygenase Activities of Lipoxygenase-1 Preparations

Enzyme	Number of -SH groups	Activity[a]
Native enzyme	4.6	232
Native enzyme + NaHS	4.5	192
Methylmercury-modified enzyme	1.5	45
Methylmercury-modified enzyme + NaHS	4.5	200

[a] μ*M* oxygen/min.

TABLE XI Co-oxidation of Some Lipoxygenases

Lipoxygenase	Oxygenation[a]	Co-oxidation[b]	Ratio (Co-ox./ oxyg.)
Modified (soybean, type-1)	20.9	6.6	0.32
Native (soybean, type-1)	132	0.4	0.003
Native (pea)	0.18	0.04	0.22

[a] μmol oxygen/min/mg.
[b] μmol crocin/min/mg.

in the oxygenation of linoleic acid by the methylmercury-modified enzyme is demonstrated by the results in Table XII [*21*].

XI. LIPOXYGENASE-MEDIATED CONVERSIONS OF FATTY ACID HYDROPEROXIDES

Lipoxygenase is not only involved in the production of hydroperoxides from fatty acid substrates, it is also capable of catalyzing the conversion of fatty acid hydroperoxides into compounds that are probably less harmful to the cell. One such example is the anaerobic reaction in which unsaturated fatty acid hydroperoxides are efficiently metabolized [*5, 10,11*]. According to the proposed mechanism of this reaction [*11*] linoleic acid serves to reduce the ferric form of lipoxygenase to a ferrous form. Although linoleic acid is a required component in this reaction it has been shown by Grosch *et al.* [*119*] and by Streckert and Stan [*120*] that linoleic acid hydroperoxides may be converted by lipoxygenase in the presence of guaiacol to a mixture of compounds consisting mainly of 9- and 13-oxooctadecadienoic acids. Galliard and Matthew [*121*] have reported on the conversion of 9-D-hydroperoxylinoleic acid into a divinyl ether derivative. Apparently, the isomeric 13-L-hydroperoxylinoleic acid is not converted in this reaction. The metabolism of fatty acid hydroperoxides in soybeans seems particularly puzzling since no well-defined protein entity other than lipoxygenase itself has been found capable of catalyzing this type of reactions in a specific way. Gardner and Kleiman

TABLE XII Stereospecificity of Modified and Nonmodified Soybean Lipoxygenase-1

Lipoxygenase	13-L-(*S*)-LOOH	13-D-(*R*)-LOOH	9-L-(*R*)-LOOH	9-D-(*S*)-LOOH
Modified	64.5	10.5	12.5	12.5
Native[a]	94.6	2.9	1.0	1.5

[a] From Van Os *et al.* [*42*].

[*122*] have reported the conversion of 13-hydroperoxylinoleic acid by a crude extract from soybeans. Among the numerous products one was identified as 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid. The latter compound can also be formed by a model system containing a mixture of $FeCl_3$ and cysteine as catalyst [*123*]. The nature of the catalytically active component in the crude soy extract has not yet been identified. Therefore, this may be an example of a reaction which does not involve lipoxygenase but instead some other enzyme system. For a recent review of enzymic reactions of fatty acid hydroperoxides see Gardner [*124*].

Verhagen *et al.* [*125*] studied the reactions of pure soybean lipoxygenase-1 with either 9-D- or 13-L-hydroperoxylinoleic acid. Under anaerobic conditions both hydroperoxides were found to be converted more than 80%. About 40% of the initial amount of hydroperoxide was converted into oxodienes absorbing at 285 nm. Typically, these reactions required a 20- to 40-fold higher enzyme concentration than the anaerobic conversion of 13-hydroperoxylinoleic acid in the presence of linoleic acid. Throughout, the 9-D-isomer appeared to be the best substrate in this reaction. The presence of a kinetic lag phase in all experiments had to be ascribed to traces of oxygen. During this lag time another enzymic reaction is taking place namely between oxygen and the fatty acid hydroperoxide. In a subsequent study this novel reaction was analyzed in more detail [*126*]. In contrast to the anaerobic conversion, this reaction did not yield chain fission products like 13-oxotridecadienoic acids. Besides epoxyhydroxymonoenoic fatty acids, which are also formed under anaerobic conditions, in the presence of oxygen a relatively large amount (> 60%) of more polar material was found.

Certain acetylenic fatty acids are inhibitors of the normal aerobic oxygenation of linoleic acid by lipoxygenase-1 [*113–115*]. However, in the aerobic and anaerobic conversions of hydroperoxylinoleic acids as described above, the acetylenic analogue of linoleic acid is a very powerful stimulator [*99*].

Egmond and Williams [*127*] have studied the conversion of 13-hydroperoxylinoleic acid with 270 MHz ^{1}H-nmr by monitoring the intensities of the C—H resonances of the hydroperoxide with time and by uv absorption spectroscopy. The results from these two methods were found to be in good agreement.

A variety of adverse effects, including those of lipid oxidation in the processing and storage of foods may have some relation to lipoxygenase activity. For example, the development of a bitter taste in ground cereals and soybeans is due to the formation and conversion of lipid hydroperoxides [*128,129*]. Fatty acid hydroperoxides are particularly suitable as

initiators of free-radical chain reactions in the presence of trace amounts of transition metals. For an authoritative treatment of this and related subjects the reader is referred to ref. [*130*]. The nature of the damage as a result from free-radical reactions in biological systems is diverse and comprises protein–protein cross-linking, the formation of lipid–protein complexes, and modifications of amino acid residues like histidine, cystine/cysteine, methionine, lysine, tyrosine, and tryptophan [*131–133*].

XII. PHYSIOLOGICAL ROLE OF LIPOXYGENASES

The widespread occurrence of lipoxygenases in both the plant and animal kingdom has evoked many hypotheses concerning a physiological role for this type of enzyme. In plant systems lipoxygenase is thought to be active at various stages during germination [*134*] and growth [*135*]. Both plant and animal systems are capable of producing α- and γ-ketols from hydroperoxylinoleic acids [*136–139*]. Also, the formation of cyclic compounds has been reported in plant systems [*56,72,140,141*]. It has been postulated further that lipoxygenase is involved in the production of a wound hormone, traumatic acid. Zimmerman and Coudron [*142*] have reported that a compound with wound hormone activity is in fact the closely related compound 12-oxo-*trans*-10-dodecenoic acid which may be a direct precursor of traumatic acid. The formation of this compound was catalyzed by extracts from runner beans and germinating watermelon seedlings [143]. Only few hydroperoxide-metabolizing enzymes have been investigated with respect to their substrate and/or product specificities. The hydroperoxide isomerase from flaxseed shows a clear preference for the 13-hydroperoxide over the 9-isomer [*144,145*] whereas the hydroperoxide-metabolizing system from potato tuber specifically utilizes the 9-hydroperoxide as the substrate [*146*]. The existence of a lipoxygenase in animal tissues has only recently been established [*52,53,147*]. Nevertheless, several possibilities for a physiological function have already been put forward. Hidaka and Asano [*148*] reported that human platelet guanylate cyclase activity is stimulated by unsaturated fatty acid hydroperoxides. It has been found by Turner *et al.* [*149*] and by Goetzl *et al.* [*150*] that 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid, which is formed by a lipoxygenase-catalyzed conversion of arachidonic acid in blood platelets [*52*], shows chemotactic activity for human polymorphonuclear leukocytes. The role of platelet lipoxygenase might be complementary to the cyclooxygenase system in providing chemotactic, vaso-active and platelet-aggregating factors during hemostasis. Hemler

et al. [*151*] showed that hydroperoxides formed in the oxygenation of polyunsaturated fatty acids by lipoxygenase can trigger ferriheme cyclooxygenase and therefore indirectly regulate prostaglandin biosynthesis. Kelly *et al.* [*152,153*] have provided some evidence for the involvement of products of the lipoxygenase-catalyzed oxygenation of arachidonic acid in the modulation of mitogenesis in human lymphocytes. Recently, it has been found that in the course of maturation of rabbit reticulocytes to erythrocytes, a characteristic lipoxygenase is synthesized [*154*]. This lipoxygenase, purified and characterized by Rapoport *et al.* [*155*] appears to be responsible for degradation of mitochondria by peroxidation of lipids and inhibition of various enzyme activities in the respiratory chain.

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