Evidence of Enzymic Production of 9-Hydroperoxy-*trans*-10,*cis*-12-octadecadienoic Acid by Peanut Lipoxygenase

Harold E. Pattee* and John A. Singleton

Four hydroperoxy geometrical isomers have been separated as methyl hydroxylinoleates by high-performance liquid chromatography with a Partisil-10 column and the optical rotations and optical rotatory dispersion (ORD) curves for selected methyl hydroxylinoleates determined, and the effects of selected reaction parameters—pH and O_2—on the enzymatically and nonenzymatically produced components of the peanut lipoxygenase–linoleic acid reaction are shown. Methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate and methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate both have positive ORD curves and the $[\alpha]^{20}_{546}$ values are +4.2° (*c*, 4.17% absolute ETOH) and +15.2° (*c*, 0.86% absolute ETOH), respectively. A pH 8.3 reaction produces only 10% as much reaction product as the pH 6.2 reaction and the relative distribution of products decreased from 7:1 to 2:1. Similarly, the ratio decreased when O_2 in the reaction was decreased.

Results of studies on the hydroperoxide isomers produced by peanut lipoxygenase (EC 1.13.11.12) have been conflicting. St. Angelo et al. (1972) suggested that 13-hydroperoxy-9,11-octadecadienoic acid (13-LOOH) is the only hydroperoxy product of the reaction. Pattee and Singleton (1977) used high-performance liquid chromatography (LC) to separate the methyl esters of the hydroperoxides and isolated the 13-LOOH (cis,trans) and 13-LOOH (trans,trans) geometrical isomers. They also isolated one 9-hydroperoxy-10,12-octadecadienoic acid (9-LOOH) and identified it as 9-LOOH (trans,trans). Although they isolated additional hydroperoxide products only the 13-LOOH (cis,trans) isomer could be considered an enzymic product. Recently Chan and Levett (1977) separated the two geometrical isomers of both 13-LOOH and 9-LOOH as their methyl hydroxy derivatives, using LC with a Whatman Partisil-5 column. This improved methodology thus makes possible in-depth studies of the enzymic formation of hydroperoxide isomers.

We now report the separation of the four hydroperoxy geometrical isomers as methyl hydroxylinoleates by LC with a Partisil-10 column, the optical rotations and optical rotatory dispersion (ORD) curves for selected methyl hydroxylinoleates, and the effects of selected reaction parameters—pH and O_2—on the enzymatically and nonenzymatically produced components of the peanut lipoxygenase–linoleic acid reaction.

MATERIALS AND METHODS

Peanut lipoxygenase was obtained from acetone powders of peanuts and purified through Sephadex G-150 as described previously (Pattee et al., 1974). High purity, grade III linoleic acid was obtained from Sigma Chemical Co. (St. Louis, MO); *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide, from Eastman Organic Chemicals (Rochester, NY); and high-purity hexane, from Phillips Petroleum Co. (Bartlesville, OK). All other solvents were commercially available reagent grades. All solvents except anhydrous diethyl ether and absolute ethanol were purified by appropriate chemical methods and distillation for removal of carbonyl contaminates and 234-nm absorbing materials. Substrate was prepared just prior to use, as 72 nmol/mL of 0.1 M borate (pH 10.2).

The enzyme reaction, product isolation, and methylation were carried out as described previously (Pattee and Singleton, 1977). The methyl hydroperoxylinoleates were reduced to hydroxylinoleates (LOH) with an excess of $NaBH_4$ in 50% MEOH–0.5 M borate buffer (pH 9.0).

LC was carried out at ambient temperature with a stainless steel column (9.4 i.d. × 500 mm) of Partisil-10. The instrument was a Varian Model 8500 attached to a Varichrom spectrophotometer. The areas under the absorption peaks at 234 nm were integrated by an Autolab System I computing integrator. The eluting solvent varied from 0.26–0.4% absolute ethanol in hexane depending on changing polarity of the column; and the solvent was delivered at 10 mL/min. Appropriate blank runs were used to correct all UV absorptions.

Infrared (IR) spectra were recorded with a Perkin-Elmer Model 521 spectrometer. The isolated methyl hydroxylinoleate isomers were filmed onto CsBr plates for the analysis.

The specific optical rotation of each methyl hydroxylinoleate was measured in the microcell of a Perkin-Elmer Model 141 polarimeter. ORD curves were made by a JASCO Model ORD/UV-5 optical dispersion recorder.

The effect of O_2 availability on the distribution of hydroperoxides was determined under four experimental conditions: (1) standardized conditions as previously described (Pattee et al., 1974), (2) saturating the system with O_2 prior to enzyme addition and rapid stirring following enzyme addition to incorporate O_2 into the reaction mixture, (3) saturating the system with O_2 prior to enzyme addition and stirring slowly following enzyme addition to avoid surface turbulence, and (4) no supplemental O_2 and slow stirring to avoid surface turbulence. In O_2 availability conditions 2–4 the reaction vessel was sealed with a ground-glass stopper after enzyme addition.

RESULTS AND DISCUSSION

Development of LC methodology capable of separating the four isomers of the lipoxygenase–linoleate reaction (Chan and Levett, 1977) has made possible conclusive studies regarding enzymic hydroperoxide production by peanut lipoxygenase. A typical LC chromatogram of the methyl hydroxy derivatives of hydroperoxides isolated from the peanut lipoxygenase–linoleate reaction shows four major compounds that absorb at 234 nm (Figure 1). Previously, the methyl hydroperoxide positional isomers from the peanut lipoxygenase–linoleate reaction were identified by mass spectrometry, gas chromatographic comparisons, and IR spectroscopy (Pattee and Singleton, 1977). These identification analyses, as well as the extensive ones of the isolated methyl hydroxylinoleates by

U.S. Department of Agriculture, Science and Education Administration, and the Department of Botany, North Carolina State University Raleigh, North Carolina 27650.

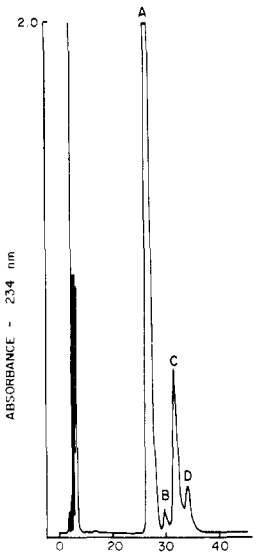

Figure 1. High-performance liquid chromatography of methyl hydroxylinoleate derivatives of hydroperoxides produced during incubation of peanut lipoxygenase with linoleic acid. Peak A: methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate; peak B: methyl 13-hydroxy-*trans*-9,*trans*-11-octadecadienoate; peak C: methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate; peak D: methyl 9-hydroxy-*trans*-10,*trans*-12-octadecadienoate.

Chan and Levett (1977), made further extensive identification work unnecessary. Absolute geometrical configurations of the isolated methyl hydroxylinoleates were determined by IR spectral analysis (Figure 2). Absorption bands at 945 and 980 cm^{-1} confirmed the cis,trans configuration of compounds identified as A and C in Figure 1. A single absorption band at 987 cm^{-1} for B and 983 cm^{-1} for D confirmed their trans,trans configuration. Thus, the four compounds in Figure 1 were assigned the following structures: (A) methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, (B) methyl 13-hydroxy-*trans*-9,*trans*-11-octadecadienoate, (C) methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate, (D) methyl 9-hydroxy-*trans*-10,-*trans*-12-octadecadienoate. The distribution of the identified compounds (Figure 1) was 83.9, 1.2, 12.5, and 2.3%, respectively. The present distribution of the trans,trans components is in excellent agreement with Hamberg (1971), who is using a gas–liquid chromatography method for separating diastereoisomeric derivatives of the hydroperoxide products of the linoleic acid–soybean lipoxygenase reaction showed a distribution of 86:4:4:6, respectively. Autoxidation of linoleic acid at room temperature with O_2, isolation of the products, and LC of the methyl hydroxylinoleates produced a distribution of 31.1, 20.9, 26.5, and 21.5%, respectively. Thus, we concluded that the distribution of the methyl hydroxylinoleates accurately reflects the distribution of the hydroperoxylinoleates formed in the peanut lipoxygenase–linoleate reaction and is not an artifact of isolation.

The higher percentage of 9-LOOH (trans,cis) than a 9-LOOH (trans,trans) suggests that the former arises by a pathway different from chemical isomerization or autoxidation. However, these would probably be the primary pathways for the formation of the two trans,trans isomers. Veldink and co-workers (1970) have shown by optical activity that soybean lipoxygenase (pH 9) can enzymatically produce both the 13- and 9-hydroperoxide positional isomers. Possibly, therefore, the 9-hydroxy-*trans*-10,-*cis*-12-octadecadienoate produced in the presence of peanut lipoxygenase was formed enzymatically contrary to previously published observations (St. Angelo et al., 1972).

Table I. Specific Rotations $[\alpha]^{20}$ of Methyl Hydroxylinoleate Derivatives of Hydroperoxides Isolated from the Peanut Lipoxygenase-Linoleate Reaction

	13-LOH[a]		9-LOH[b]		
	cis,trans[c]	trans, trans	trans,cis		trans, trans
λ, nm	0.86	0.9	4.17	2.08	2.0
589	+11.0	0		+2.4	0
578	+12.8	0	+3.5	+2.9	0
546	+15.2	Tr	+4.2	+3.5	
436	+29.7	Tr	+3.5		Tr

[a] Methyl 13-hydroxy-9,11-octadecadienoate. [b] Methyl 9-hydroxy-10,12-octadecadienoate. [c] Numbers below isomeric designations indicate percentage concentrations in absolute ethanol.

To firmly establish which hydroperoxide(s) arose enzymatically, we determined the optical rotations of their hydroxy derivatives (Table I). The components upon re-LC were 99% pure, as indicated by their 234-nm absorbance profiles prior to the optical rotation determinations. The optical activities of both the 13-LOH (cis,trans) and 9-LOH (trans,cis) derivatives indicates that 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate and 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate were enzymatically formed. Hence, peanut lipoxygenase, like soybean lipoxygenase, enzymatically produces both the 13- and 9-cis,trans isomers.

The $[\alpha]^{20}_{546}$ +4.2° and +3.5° values listed in Table I agree closely with the optical rotation $[\alpha]^{20}_{546}$ +5.3° (*c*, 3.25% hexane) obtained by Gardner and Weisleder (1970) for methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate produced by corn lipoxygenase. The observed rotation also agrees, both in direction and magnitude, with the $[\alpha]^{25}_{589}$ +5° (*c*, 5% chloroform) obtained for methyl 9-hydroxy-*cis*,*trans*-octadecadienoate from *Calendula* oil (Smith et al., 1960).

Both trans,trans isomers showed very little optical rotation ($[\alpha]^{20}$ <0.2°) as compared with the comparable solutions of the cis,trans isomers; hence, the former isomers likely were not found enzymatically. Chan et al. (1975) suggested that the trans,trans components were isomerization products of the cis,trans components; however, we found that reductions in reaction time (5 s) and LC preparation time (72–24 h) did not affect the percentage distribution of the isomers. Failure to control chemical isomerization during sample preparation for IR analysis, however, must have caused the observation by Pattee and Singleton (1977) that the predominant 9-LOOH positional isomer was trans,trans. The problem of chemical isomerization is greatly reduced if the hydroperoxide is converted to the more stable hydroxy acid. However, we have found that even the hydroxy acids, especially their concentrated solutions, should not be exposed to room temperature for extended periods. Such a precaution has been pointed out by Chan and co-workers (1975, 1977) for hydroperoxy solutions. The agreement between trans,trans distribution values of Hamberg (1971) and our distribution values is additional evidence that minimum autoxidation and chemical isomerization had occurred during the reaction and isolation periods.

The optical rotatory dispersion (ORD) curves for 9-LOH (trans,cis) and 13-LOH (cis,trans) were both positive (Figure 3). Using the reasoning applied by Gardner and Weisleder (1970), Veldink et al. (1970), and Hamberg and Samuelsson (1967), we found that, in terms of their absolute configurations, the enzymatically produced hydroperoxides were 9-D-hydroperoxyoctadecadienoic acid

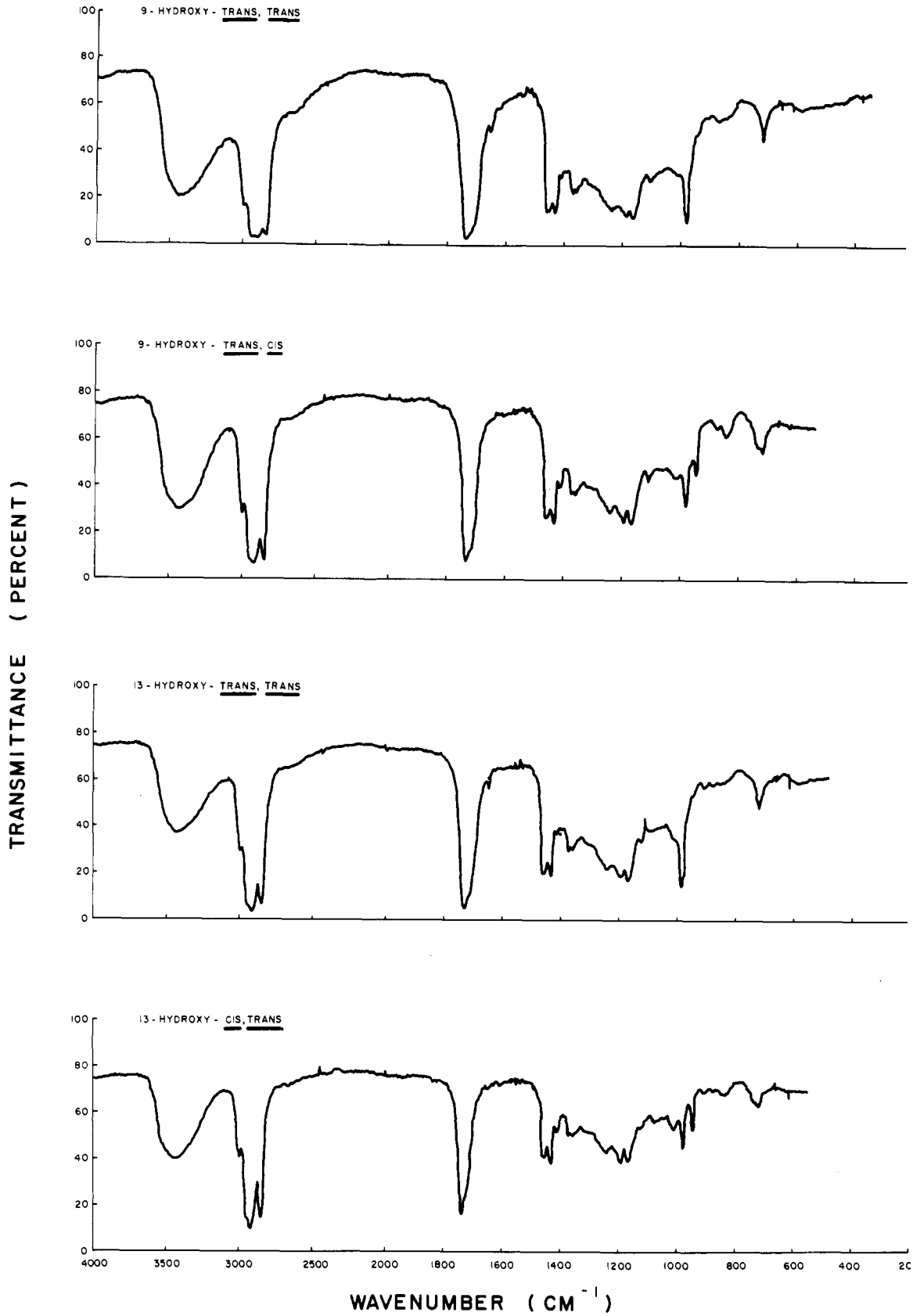

Figure 2. Infrared spectra of methyl hydroxylinoleate derivatives of hydroperoxides produced during incubation of peanut lipoxygenase and linoleic acid.

and 13-L-hydroperoxyoctadecadienoic acid.

Since enzyme source and reaction conditions influence the percent distribution of reaction products (Christopher et al., 1972; Galliard, 1974; Galpin and Allen, 1977; Singleton et al., 1978), and having established that peanut lipoxygenase produces two hydroperoxides from linoleic acid, we investigated the effects of reaction pH and O_2 concentration on the relative distribution of the two products. The alternate pH of 8.3 was chosen because it is the pH optimum of one of the peanut lipoxygenase isozymes (Sanders et al., 1975). Table II shows that at pH 8.3 only 10% as much reaction product was formed and

Table II. Effect of Reaction pH on the Distribution of Hydroperoxides (Analyzed as Their Methyl Hydroxy Derivatives) Formed in the Peanut Lipoxygenase-Linoleate Reaction

reaction pH	LOH, mg	13-LOH[a] cis,trans mg	%	13-LOH trans,trans mg	%	9-LOH[b] trans,cis mg	%	9-LOH trans,trans mg	%
6.2	93.3[c]	73.2	78.4	3.4	3.7	11.8	12.6	4.9	5.3
8.3	9.8	4.9	50.3	0.9	9.6	2.8	29.0	1.1	11.1

[a] Methyl 13-hydroxy-9,11-octadecadienoic acid. [b] Methyl 9-hydroxy-10,12-octadecadienoic acid. [c] Average of two replications and two LC injections.

Table III. Effect of Oxygen Availability on the Distribution of Hydroperoxides (Analyzed as Their Methyl Hydroxy Derivatives) Formed in the Peanut Lipoxygenase-Linoleic Acid Reaction

reaction condition	total mg of LOH	13-LOH[a] (cis,trans) mg	%	13-LOH (trans,trans) mg	%	9-LOH[b] (trans,cis) mg	%	9-LOH (trans,trans) mg	%
pH 6.2 standard	93.3[c]	73.2	78.4	3.4	3.7	11.8	12.6	4.9	5.3
O_2 before enz., O_2 incorporation by stirring	79.4	53.8	67.8	4.8	6.0	15.2	19.2	5.6	7.0
O_2 before enz., slow stirring, no O_2 incorporation	55.5	34.6	62.3	4.3	7.8	11.7	21.0	4.9	8.9
No O_2 before enz., slow stirring, no O_2 incorporation	24.3	12.2	50.2	2.8	11.3	6.7	27.4	2.7	11.1

[a] 13-Hydroperoxy-9,11-octadecadienoic acid. [b] 9-Hydroperoxy-10,12-octadecadienoic acid. [c] Average of two replications and two LC injections.

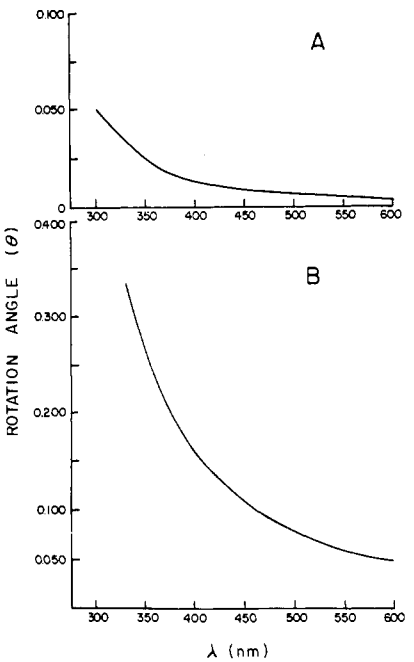

Figure 3. Optical rotatory dispersion curves of: (A) methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate; (B) methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate. Solvent, absolute ethanol.

that the relative distribution of products was significantly changed, the 13-LOOH to 9-LOOH ratio of enzymatically produced components decreasing from 7:1 to 2:1. Similarly, the ratio decreased when O_2 in the reaction was decreased (Table III). These results suggest that the mechanism of the reaction changes as the available O_2 concentration level decreases or as to the ratio of active enzyme (pH 8.3) to substrate becomes inhibitory. Egmond and co-workers (1977) have discussed in detail the kinetics involved in the formation and conversion of yellow and purple soybean lipoxygenase-1 species, as influenced by substrate and available O_2 levels. We speculate that reaction conditions involving high linoleates to O_2 ratios, and which produce the noted changes (Egmond et al., 1977) in enzyme conformation and oxidative states, may also change the positional specificity of the enzyme or promote the randomness of substrate attachment. Hamberg and Samuelsson (1967) showed that the 13-L-hydroperoxide formation by soybean lipoxygenase involves the stereospecific abstraction of the L_S hydrogen from carbon 11. On the other hand, Egmond et al. (1972) showed that removal of the D_R hydrogen at C-11 results in the formation of 9-D-hydroperoxide by corn lipoxygenase. Thus, the stereospecific abstraction of a C-11 hydrogen determines the hydroperoxide isomer formed and has been proposed by Hamberg (1971) as a possibility accounting for the formation of 9-D-LOOH. However, he was unable to obtain a pure 9-D-LOOH fraction by his technique and thus could not determine if it was a lipoxygenase product. The LC procedure does provide a pure sample which can be used for many avenues of product analysis including the steric analysis technique of Hamberg (1971). With peanut lipoxygenase, the tendency is toward the formation of the 13-L-hydroperoxide; but conditions which inhibit the reaction also decrease the hydrogen abstraction selectivity of the enzyme. In support of this conclusion is the finding (Nelson et al., 1977) that when the reaction is run at pH 8.3 addition of calcium not only increases product formation but also increases the 13-LOOH to 9-LOOH ratio. However, we have not been able to increase the ratio beyond 85:11 using peanut lipoxygenase. Also recent work by Galpin and Allen (1977) on the influence of micelle formation on lipoxygenase kinetics seems to suggest that we should investigate the physiochemical state of the substrate in relation to the conformational state of the isozyme as a major factor influencing the product specificity of the reaction rather than ascribing the product specificity solely to the nature of the lipoxygenase isozyme.

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Lipid Hydroperoxide Reactivity with Proteins and Amino Acids: A Review

H. W. Gardner

Lipoxygenase is responsible for the production of lipid hydroperoxides in inadequately processed foods. The hydroperoxides as well as their products of decomposition are potentially reactive substances that can cause deterioration of food proteins or amino acids. Among the many consequences of protein exposure to peroxidized lipids is the formation of lipid–protein complexes that are bound through purely physical forces. Chemical changes caused by interaction of lipid hydroperoxide and protein are protein–protein cross-links, protein scission, protein–lipid adducts, and amino acid damage. The secondary products arising from hydroperoxide decomposition also readily damage protein and amino acids through formation of covalent bonds. Among the secondary products, aldehydes have received the most attention because of their propensity to form Schiff base adducts with amino groups, and, in particular, the bifunctional malondialdehyde can cross-link protein via Schiff base formation.

It is remarkable that living tissue is relatively immune to lipid peroxidation although all the necessary ingredients are present, i.e., polyunsaturated lipids, O_2, and catalysts. The resistance to lipid oxidation attests to the precise cellular compartmentalization by which a high degree of organization protects the cell from self-destruction. When tissue is disrupted as encountered in the isolation of cellular organelles, lipid oxidation is initiated, and this eventually leads to damage of membrane structure (Mead, 1976; McKnight et al., 1965; Robinson, 1965) and inactivation of enzymes (Bernheim et al., 1952). In particular, protein in biomembranes exists in close molecular proximity to polyunsaturated lipids, thus membrane protein would be readily damaged by lipid oxidation. In many foodstuffs the cellular integrity has been disrupted in varying degrees. As a result, care must be exercised to prevent lipid oxidation and attendant loss of quality and flavor. If large quantities of lipid peroxides accumulate in food, they can be toxic when ingested (Barber and Bernheim, 1967). Protein and amino acids are only one class of biochemicals in foods that is susceptible to the damaging effects of lipid hydroperoxides.

Northern Regional Research Center, Federal Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Illinois 61604.

The terminologies, lipid peroxides, peroxidized lipid, lipid hydroperoxides, are used interchangeably in the text. The term "peroxide" strictly is inaccurate nomenclature, but it is one that has gained wide usage. For the most part, hydroperoxides are the initial products of lipid oxidation and usually account for the majority of bound oxygen measured by peroxide value. Various endoperoxides and peroxides are usually secondary products and probably amount to a relatively small part of the total. In the text the term "peroxidizing" is distinguished from "peroxidized" since a lipid actively in the process of oxidation may affect protein or amino acids differently than a lipid that already had been peroxidized. Also, the term "peroxidized" should be distinguished from a specific isolated lipid hydroperoxide, such as linoleic acid hydroperoxide, because a "peroxidized" lipid implies an impure mixture containing hydroperoxides, unoxidized lipid, and secondary products.

The interaction between lipid hydroperoxide and protein/amino acids is complex indeed, a complexity that is contributed to by both lipid hydroperoxide and its secondary products of decomposition. In one such interaction, the lipid hydroperoxide and/or secondary products will physically complex with protein. In another, various types of covalent bonds are the result. These chemical reactions are categorized as to type in the text below. Other reviews that cover different aspects of the