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Department of Food Science and Human Nutrition
Washington State University
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CHAPTER 6

LIPOXYGENASE PATHWAY IN CEREALS

H. W. GARDNER
Northern Regional Research Center
U.S. Department of Agriculture
Agricultural Research Service
Peoria, Illinois

I. INTRODUCTION

Although many reviews on enzymatic lipid oxidation have been published, few have dealt specifically with the role of enzymes in the oxidation of cereal lipids. Morrison’s (1978) excellent treatise on cereal lipids included an overview of cereal lipid oxidation, and his chapter is recommended reading. The current review examines specifically the lipoxygenase (LOX) pathway, which includes the action of a number of sequential enzymes reactive with the product of LOX action. Research on this topic has expanded considerably over the last few years.

Cereal lipids are rich in polyunsaturated fatty acids, making them substrates for LOX action. In general, linoleic acid makes up about half the total fatty acid composition in cereals, and linolenic acid amounts to about 5 ± 3% (Morrison, 1978). Since these fatty acids contain a pentadiene moiety, they are readily oxidized by cereal LOXs into conjugated hydroperoxydiene fatty acids. The hydroperoxides are often transformed by other enzymes into a variety of aldehydes and oxygenated fatty acids. These end products are often detrimental to cereal quality, and additionally, may play a role in the physiology of the cereal plant.

II. SUBCELLULAR LOCALIZATION OF LIPIDS

To fully understand enzymatic lipid peroxidation, it is important to localize both lipids and lipid-active enzymes within tissues and cells. Lipid location is briefly described here, but discussion of lipid-active enzymes is mainly relegated to later sections.

Cereal germ is generally the tissue containing most of the lipid, as well as the lipid-active enzymes. The germ contains about 10–40% lipid by weight, of which the major component is triglycerides (Morrison, 1978). Although germ makes up a small portion of the whole kernel (1.5–14%), most of the triglyceride lipid from the whole grain originates in this oil-rich tissue. Oats, the exception among
grains, has its lipid, including triglyceride, localized mainly in the endosperm (Morrison, 1978). Within germ cells, triglyceride is packaged into oil bodies or oleosomes. Oil bodies have often been confused with spherosomes, but recently investigators have categorized spherosomes as separate organelles packed with protein and phospholipids (Gurr, 1980). Oil bodies, oil droplets surrounded by a half-unit membrane (Yatsu and Jacks, 1972), are found throughout specific parts of the germ, that is, the embryo, coleoptile, scutellum (Morrison, 1978) and coleorhiza (Frey-Wyssling et al, 1963).

Besides triglycerides, additional lipids, such as glycolipids, phospholipids, and unsaponifiable lipids are found in the germ. Presumably they originate from various membranes, such as the half-unit membrane of oil bodies, plasma membranes, and other organelles. In wheat germ (Mecham, 1971) and maize germ (Tan and Morrison, 1979), phospholipids and glycolipids amount to 16.5 and 5%, respectively, of the total lipid. A major portion of the kernel glycolipids and phospholipids is found in the germ (Morrison, 1978; Tan and Morrison, 1979; Weber, 1979).

Spherosomes in the aleurone layer cluster around the subcellular bodies called aleurone grains (Buckout et al. 1981). In certain cereals, such as wheat (Wilkinson et al, 1984), the aleurone spherosomes are rich in triglycerides, possibly indicating that they are not true spherosomes. Maize aleurone also contains a high content of triglyceride (Tan and Morrison, 1979).

Except for oats, cereal endosperm is relatively deficient in lipids, 2% or less by weight for most cereals (Morrison, 1978). In mature wheat and maize endosperm, the lipid comprises a complex mixture of phospholipids, galactolipids, neutral lipids (especially free fatty acids), lysophospholipids, lysogalactolipids, and acylated lipids, such as N-acyl-phosphatidylethanolamine and esterified galactolipids (Morrison, 1978; Tan and Morrison, 1979; Weber, 1979). The lipid pattern of developing endosperm is markedly different and is notably lacking in the acylated and lyso varieties of phospholipid and galactolipid (Weber, 1970). It is reasonable to assume that the lipids of developing endosperm should reflect the composition of the major organelles of this tissue. The cells of developing endosperm possess the usual complement of subcellular organelles, but they are dominated by amyloplasts, protein bodies, and ribosome-rich endoplasmic reticulum (see, for instance, Liu and Shannon, 1981). The latter three organelles are readily identified in mature endosperm as well (Simmonds, 1972). Protein bodies contain inclusions of lipids (Morrison, 1978), and in rice this lipid contains primarily free fatty acids and triglyceride (Mano, 1982). According to Mudd (1980), plant endoplasmic reticulum is composed of phospholipids, of which phosphatidylycholine and phosphatidylethanolamine predominate. Analysis of maize amyloplast membranes from the developing endosperm revealed predominantly digalactosyldiglyceride followed by monogalactosyldiglyceride, phosphatidylycholine, phosphatidylinositol, and phosphatidylglycerol (Gardner et al, 1984a). The starch granule contained within the amyloplast is composed of 0.5–1% lipid, which is largely free fatty acid and lysophosphatidylycholine (Morrison, 1978; Tan and Morrison, 1979). The granule lipids exist as an inclusion complex with amyllose and are likely present at the time of amyllose biosynthesis (Morrison, 1978). Since lyso- and acylated galactolipids, and N-acyl phosphatidylethanolamine are not present in immature endosperm, these compounds probably arise after grain maturity.
from deterioration of organelle membranes.
Understanding the localization of lipids in cereal grains establishes a basis for understanding the oxidation of lipids in cereals.

III. SEQUENTIAL ENZYMATIC REACTIONS

Endogenous lipids of cereals are oxidized by a cascade of enzymatic reactions (Figure 1). The sequence begins with hydrolysis of various glyceride lipids by lipases, phospholipases, and lipolytic acyl hydrolases; the polyunsaturated fatty acids freed by lipolysis are then susceptible to oxidation by LOX. Most of the known LOXs utilize free fatty acids as substrates; however, certain isoenzymes, such as LOX-2 from soybeans, oxidize triglyceride fatty acid (Christopher et al., 1970). The products of LOX, fatty acid hydroperoxides, are then transformed into oxygenated fatty acids and aldehydes as shown in Figure 1. Food technologists are interested in this sequence primarily to control off-odors and bitter tastes (Gardner, 1985a). This “linoleic/linolenic acid cascade” may also be important to the physiology of plants (Gardner, 1985b; Vick and Zimmerman, 1987).

Because lipid hydrolysis is first in the sequence, it may have an important role in controlling lipid deterioration. Numerous reviews of lipolytic activity in cereals and other plants are found in the literature (Brockerhoff and Jensen, 1974; Galliard, 1975, 1980; Morrison, 1978; Huang, 1984); thus only selected investigations are reviewed here.

Of the cereal lipolytic enzymes, lipases appear to have received the most attention. Lipase activity normally is quiescent in mature seed, but it can be potentiated in certain cereal fractions after the tissue has been disrupted by milling or flaking. For example, lipase activity is high in oat flakes (Acker and

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**Figure 1.** The linoleic/linolenic acid cascade in cereals.
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Seed germination also increases lipase activity. Maize lipase, which is virtually absent in quiescent grain, appears upon germination, and the activity continues to increase through six days (Wang et al., 1984). Lipase is specifically associated with the lipid bodies in maize germ (Lin et al., 1983). During germination, the lipid content of maize germ is correlated inversely with both catalase and lipase activity (Lin et al., 1983). Since catalase is a marker for glyoxysomes, this correlation implies that free fatty acids released by lipase are being metabolized by glyoxysomes for conversion into carbohydrates (Beavers, 1980). Similarly, wheat lipase becomes active after germination and increases in activity over several days (Drapron et al., 1969). On a dry weight basis, the wheat coleoptile is the most active, followed by rootlets, and the residual grain has very low activity. However, on a per-kernel basis, the residual grain (endosperm and bran) has more lipase activity than the germ because of their difference in weight (Tavener and Laidman, 1972).

After lipolysis, the released free fatty acids are metabolized further by a sequence of LOX and other enzymes (Figure 1). As will become evident later, LOX, hydroperoxide isomerase, and hydroperoxide cyclase are active in mature, quiescent grains. LOX and hydroperoxide isomerase are localized mainly in the germ of maize (Gardner, 1970), wheat (Zimmerman and Vick, 1970), and barley (Yabuuchi and Amaha, 1976). Activities of LOX, hydroperoxide isomerase, and hydroperoxide cyclase increase during maize germination (Vick and Zimmerman, 1982), generally parallel with the increase in lipase activity (Lin et al., 1983). Although the primary function of lipolysis in germinating seeds is to provide free fatty acids for metabolism into carbohydrates by glyoxysomes, it is not known how much of the polyunsaturated fatty acids are diverted into the linoleic-linolenic acid cascade. According to Vick and Zimmerman (1982), small quantities of the cascade end-products, α-ketols and 12-oxophytodienoic acid (12-oxo-PDA), can be detected in intact maize seedlings. Furthermore, cellular disruption increased the levels of α-ketols and 12-oxo-PDA. Apparently, the cascade of lipid deterioration caused by tissue disruption is a phenomenon of general distribution in plants (Galliard, 1970, 1975).

Lipid enzymes of intact cereal grains in the dry state ordinarily are inactive, but they can be activated simply by tissue disruption in the apparent absence of water. However, enzyme activity in “dry” cereals is dependent on moisture content. Inasmuch as the relative humidity of the ambient atmosphere determines the moisture content of solid substrates, water adsorption isotherms have been constructed for various cereals (Hubbard et al., 1957; Shelef and Mohsenin, 1966). At constant relative humidity, the moisture contents of different substrates often vary considerably; thus, a useful description of water activity is via the relative humidity of ambient atmosphere. Lipase activity in oat flakes increased when the flakes were equilibrated under increasing relative humidities, but lipolysis persisted at as low as 10% relative humidity (Acker and Beutler, 1965). In this study, a remarkable parallel between lipase activity and the water adsorption isotherm was observed. In the oat flakes, the triglyceride substrate had to be liquid to obtain significant activity, implying that liquid substrate was transported to the enzyme. On the other hand, Purr (1966) reported that lipase in low-humidity model systems could hydrolyze solid fats as
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well. Other dry-state model systems tested by Purr (1970) included combinations of lipase, phospholipase, LOX, and peroxidase. In these experiments, sequential enzyme activity was detected even at very low relative humidity, where only bound water exists. Brockmann and Acker (1977a, 1977b, 1977c) also reported LOX activity in dry model systems, and the activity increased as relative humidity was elevated. According to Gardner and Inglett (1971), lipid peroxidation in low-moisture maize germ flakes may have been prevented by hydroperoxide isomerase activity. The presence of isomerase correlated with low lipid peroxide values of oil extracted after storage of the flakes. Since enzymes suspended in organic solvents will react with their substrates (Luisi, 1985; Klibanov, 1986), it should not be surprising that enzymes are active at low moisture content. Enzymes need only sufficient moisture to surround themselves with water in reverse micelles. Both LOX and lipase will react in organic media (Luisi, 1985).

As discussed above, the initiation of sequential lipid degradation in dry, mature seeds appears to be controlled by many factors. First, cellular disruption appears to promote the release of enzymes from their specific "packages" within organelles. Second, the first enzymes of the sequence, the lipolytic enzymes, should be present to initiate the process. In many quiescent seeds, lipolytic enzymes are absent or are present at low levels. Third, if enzymes are present, they can be active even under conditions of low relative humidity; however, increased moisture results in accelerated rates of activity. Finally, seed germination causes the appearance and/or increase of lipid-degrading enzymes. Seed germination requires much more water than encountered under normal storage conditions, and indeed, storage fungi will grow on grain with lower moisture requirements than needed for germination. Infestation by storage fungi usually produces lipase activity many orders of magnitude greater than that of indigenous grain lipases (Baker et al, 1959; Christensen and Kaufmann, 1969). According to Hubbard et al (1957), a relative humidity of 75.6% is about borderline for growth of storage fungi on wheat and maize. Even with the above factors kept at a minimum, triglyceride hydrolysis can occasionally cause quality problems. Very low enzyme activity over a long period of storage has a cumulative effect.

IV. Lipoxygenase

A. General Properties

LOX is an enzyme found in both plants and animals. The enzyme catalyzes the oxidation by molecular O₂ of polyunsaturated fatty acids with a methylene-interrupted cis,cis-pentadiene moiety into conjugated cis,trans-hydroperoxydiene fatty acids. Numerous reviews of plant LOXs can be found (Tappel, 1963; Drapron and Uzzan, 1968; Grosch, 1972; Axelrod, 1974; Galliard, 1974, 1975; Hamberg et al, 1974; Boldingh, 1976; Eskin et al, 1977; Gibian and Galaway, 1977; Veldink et al, 1977; Vliegenthart et al, 1979; Galliard and Chan, 1980; Gardner, 1980, 1985a, 1985b; Vick and Zimmerman, 1987). The specific topic of cereal LOXs was addressed by Morrison (1978).

An overview of the known properties and mechanisms of LOX requires discussion of the isoenzymes found in soybean, which have been extensively
The molecular weight of plant LOXs, including the three isoenzymes in soybean, is about 100,000 ± 20,000. One mole of nonheme iron per mole of LOX was discovered by Chan (1973), and this was confirmed by Roza and Francke (1973) and Pistorius and Axelrod (1974). The iron was scavenged from LOX by chelating agents, provided that it was first exposed to thiols (Roza and Francke, 1973; Pistorius and Axelrod, 1974). Either the iron was bound by sulfide ligands, or the thiols reductively cleaved disulfide bonds, exposing the active site to the chelators. The number of half-cystine and cysteine residues reported to be present has ranged from four to 12 (Veldink et al., 1977).

As stated above, the preferred substrate of LOX is fatty acids with a methylene-interrupted cis,cis-pentadiene moiety. However, certain fatty acids that can be categorized as such are, in fact, poorly oxidized by LOX. Holman et al. (1969), who examined a series of positional isomers of octadecadienoic acids with soy LOX-1 isoenzyme, found a number of unsuitable substrates, especially those with double bonds located near the carboxylic acid group. The data of Holman et al. (1969) and Hamberg and Samuelsson (1967) indicated that the most preferred substrates of soy LOX were fatty acids with a methylene that was both situated at the \(\omega-8\) carbon and localized between two double bonds. The fatty acids commonly found in plants that fit these requirements are linoleic and linolenic acids. Although fatty acids are usually the substrate of choice, the soybean LOX-2 isoenzyme was about equally active with linoleic acid, methyl linoleate, and trilinolein (Christopher et al., 1970).

Considerable chiral and positional control occurs in the placement of \(\mathrm{O}_2\) in fatty acids by LOX. With linoleic and linolenic acids, the placement of the hydroperoxide group occurred either at carbon-9 or carbon-13, but the chirality of oxidation was nearly always S (Figure 2). The various LOX isoenzymes displayed different behavior in oxidizing the fatty acids with these two different positions, and often the products were influenced by reaction conditions. Depending on the pH, \(\mathrm{O}_2\) concentration, and temperature, soybean LOX-1 oxidized linoleic acid to mixtures that varied considerably in the isomeric ratio of 13-hydroperoxy-cis-9,trans-11-octadecadienoic acid (13-LOOH) to 9-

![Figure 2. The oxidation of linoleic and linolenic acids by lipoxygenase.](image-url)
hydroperoxy-trans-10,cis-12-octadecadienoic acid (9-LOOH) (Christopher et al., 1972). The positional specificity of oxidation by soybean LOX-2 was also dependent on environmental conditions, but this dependence was relatively less than with LOX-1.

Abbreviations for linoleic acid hydroperoxide isomers (LOOH), linolenic acid hydroperoxide isomers (LnOOH), and hydroxyoctadecadienoic acid isomers (LOH), as well as for epoxyhydroxyoctadecenoic acid isomers (EHA) and trihydroxyoctadecenoic acid isomers (THA) are given in Table 1.

A high degree of stereospecific oxidation is also observed. Linoleic and linolenic acids were oxidized by soy LOX-1 into (13S)-13-hydroperoxy-cis-9,trans-11-octadecadienoic acid (13S-LOOH) and (13S)-13-hydroperoxy-cis-9,trans-11,cis-15-octadecatrienoic acid (13S-LnOOH), respectively (Hamberg and Samuelsson, 1967). Also, soy LOX-1 oxidized linoleic acid into a small

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percentage of (9S)-9-hydroperoxy-trans-10,cis-12-octadecadienoic acid (9S-LOOH) (Veldink et al., 1970a). In practice, the R enantiomers are also found, presumably due to autoxidation reactions (Hamberg, 1971). Thus, at pH 9, soy LOX-1 oxidized linoleic acid mainly to 13S-LOOH, but a few percent of the R-isomer (13R-LOOH) was observed. The 9-LOOH product (10-20% of the total) showed a slight preponderance for the 9S-LOOH over the R-isomer (9R-LOOH) (Hamberg, 1971; Egmond et al., 1972). At pH 6.6, oxidation of linoleic acid by soy LOX-1 led to an increased ratio of 9-LOOH to 13-LOOH and also increased the chiral preference for 9S-LOOH over 9R-LOOH (van Os et al., 1979a). These data clearly showed that soy LOX-1 was capable of forming two chiral products, 13S-LOOH and 9S-LOOH, and that the R-isomers were probably from autoxidation. Oxidation by soy LOX-2 and LOX-3 led to the formation of increased percentages of the R enantiomers, presumably because these isoenzymes are more peroxidative or free radical in character. When crocin was used to quench peroxo radicals produced by LOX-3, the percentage of both the 13R-LOOH and 9R-LOOH isomers were reduced from 32 to 22% of the total (Weber and Grosch, 1976). At its pH optimum (pH 6.6), LOX-2 afforded mainly a racemic R,S mixture of both the 9-LOOH and 13-LOOH, but at pH 9, the 13S-LOOH and 9R-LOOH isomers predominated (van Os et al., 1979b). This preference for 9R-LOOH by LOX-2 oxidation at present is the only known R specificity of plant LOXs. Although the soy LOX isoenzymes do not include one that predominates in the formation of 9S-LOOH from linoleic acid, this type of LOX is common in quiescent cereal seeds, as is discussed later.

The literature abounds with confusion over the stereochemistry of the 9S-LOOH product, which is also D by the Fischer convention. The probable reason for the confusion by several authors lies in the difference in the stereochemical rules between the Fischer and Cahn-Prelog-Ingold conventions. This difference was succinctly explained by Smith (1970) specifically regarding oxygenated, conjugated diene fatty acids.

Hamberg and Samuelsson (1967) found that because the removal of a hydrogen from the ω8 methylene of substrate fatty acids was rate limiting, this removal preceded the oxidation step. They also discovered that hydrogen removal was chiral and occurred on the opposite side of the fatty acid chain from the insertion of O2. The chiral hydrogen removal was confirmed by Egmond et al. (1972), who tested both soy LOX-1 and maize germ LOX.

Extensive mechanistic studies of soy LOX by Vliegenthart’s group in the Netherlands, Axelrod’s group in the United States, and others have resulted in a good understanding of the enzyme’s mode of action. As shown by Figure 3, under aerobic conditions the iron-active site participates in a redox cycle that utilizes substrate fatty acid as a reductant and molecular O2 as an oxidant (de Groot et al., 1973). LOX also functions in an anaerobic mode, provided that fatty acid substrate and product hydroperoxide are both present (Garssen et al., 1971, 1972; de Groot et al., 1973; Verhagen et al., 1977). In the anaerobic reaction, the fatty acid hydroperoxide replaced O2 as the oxidant of iron, and consequently the hydroperoxide group was reductively cleaved into a hydroxide ion and an alkoxy radical. As shown by Figure 3, the anaerobic LOX reaction of 13-LOOH in the presence of linoleic acid afforded end products that were characteristic of products having their origin from the alkoxy radical (Garssen et al., 1971, 1972; Gardner, 1975a).
Smith and Lands (1972) noted two important characteristics of soy LOX. The first was a kinetic lag phase that could be abolished by addition of small amounts of product fatty acid hydroperoxide. This small amount of oxidizing equivalents was necessary to “prime the pump” to obtain the full activity of the enzyme. The second characteristic was a self-catalyzed destruction of the enzyme, which apparently is a general phenomenon common to all LOXs. Smith and Lands (1970, 1972) postulated that self-destruction occurred as a result of uncontrolled free radicals generated as side products of the main event of fatty acid hydroperoxidation.

LOX in the presence of its substrate will cooxidize a number of susceptible compounds, such as carotenoids, chlorophyll, and 2,6-dichlorophenolindophenol. The cooxidation reaction is usually measured by bleaching. Cooxidation is a dynamic phenomenon arising from enzymatic action and does not occur with the addition of hydroperoxide product in the absence of fatty acid substrate (Weber et al, 1973). Most investigators believe that cooxidation occurs as a result of free radical generation by LOX during oxidation of its fatty acid substrate. Accordingly, cooxidation is inhibited by the antioxidants, \( \text{n-propyl gallate} \) (Ikediobi and Snyder, 1977) and nordihydroguaiaretic acid (Weber et al. 1973). In comparison to LOX-2 and LOX-3 of soy, LOX-1 is a weak cooxidizing enzyme, but its ability to cooxidize 2,6-dichlorophenolindophenol is considerably improved under anaerobic reaction conditions (Axelrod, 1974). As discussed above, the anaerobic reaction is particularly efficient at producing free radicals, which are possibly the cause of the observed cooxidation. LOX-2 and LOX-3 are readily able to cooxidize even under aerobic conditions (Axelrod.

Figure 3. Proposed scheme for lipoxygenase oxidation of linoleic acid under aerobic and anaerobic conditions. Fatty acid structures are abbreviated to show only C-8 through C-14. Modified from de Groot et al (1975) with permission.
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1974; Weber and Grosch, 1976; Grosch and Laskawy, 1979). LOX-2 and LOX-3 also aerobically generate anaerobic-type products, e.g. oxooctadecadienoic acid (Axelrod, 1974), indicating that these isoenzymes may be more prone to uncontrolled free radical reactions. The peroxidative nature of LOX-3 is also demonstrated by the formation of a nearly racemic mixture of hydroperoxides from oxidation of linoleic acid (Weber and Grosch, 1976). Racemization of hydroperoxides is known to occur via β-scission of peroxy radicals (Chan et al., 1978). Suppression of radicals by supplying a cooxidation substrate, crocin (a water-soluble carotenoid), increases the oxidation specificity of LOX-3 (Weber and Grosch, 1976).

B. Subcellular Localization of LOX

Because LOX can be readily solubilized by tissue homogenization, it was once thought to be randomly distributed throughout the cytosol. Recent work has complicated this view. Although most of the research in this area has been completed with plants other than cereals, a number of similarities between plant families may exist.

LOX localization in soybean seed displayed a dependence on developmental stages. When soybean cotyledons were either immunofluorescence stained with anti-LOX immunoglobulin G or indirectly labeled with a protein A-colloidal gold complex, LOX was found mainly in the cytosol of the storage parenchyma (Vernooy-Gerritsen et al., 1983, 1984). However, upon seed germination, LOX decreased in the storage parenchyma and increased in the abaxial hypodermis, epidermis, and vascular bundle sheaths (Vernooy-Gerritsen et al., 1983). In the cells of the hypodermis and vascular bundle sheath, LOX became associated with an aberrant type of protein body, unlike the protein bodies found in storage parenchyma (Vernooy-Gerritsen et al., 1984).

In pea roots, the subcellular localization of LOX was found in a particulate fraction coincident with lysosomes by sedimentation on a density gradient, but in cauliflower and calabrese florets the LOX sedimented at a density similar to that of intact and broken chloroplasts (Wardale and Galliard, 1975, 1977). Because the LOX-containing particle was not exactly identical in density to the chloroplast fraction in cauliflower and calabrese, these latter investigators believed LOX was contained in a different organelle. According to Douillard and Bergeron (1979, 1981), who studied LOX activity in wheat leaves, the slight difference in densities of the LOX-containing particles and the chloroplasts was due to a higher LOX content in the slightly more dense juvenile chloroplasts. LOX has been located in the chloroplasts of a number of other plants, such as soybean leaves (Vernooy-Gerritsen et al., 1983) and pea leaves (Haydar and Hadziyev, 1973). LOX was absent in mitochondria isolated from wheat seedlings (Goldstein et al., 1980). Although LOX of cereal seeds is primarily found in the germ, the subcellular location of the enzyme in germ tissue is unknown.

C. Wheat LOX

HISTORICAL PERSPECTIVE

Early studies established the presence of LOX in wheat and wheat flour and
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defined some of the properties of crude extracts containing LOX isoenzymes (Sumner, 1943; Miller and Kummerow, 1948; Irvine and Winkler, 1950; Franke and Frehse, 1953; Irvine and Anderson, 1953a, 1953b; Blain and Todd, 1955; Siddiqi and Tappel, 1957).

DISTRIBUTION IN THE SEED
LOX activity varies considerably in wheat according to localization in the seed, conditions of growth, variety, and extent of germination. According to Auerman et al (1971a), the germ and bran of wheat contained 17-fold and fourfold more activity, respectively, than the endosperm. Von Ceumern and Hartfiel (1984) reported a similar distribution. A greater than 10-fold range of LOX activity was found in wheat samples that differed in variety and conditions of cultivation (Colas and Chargelegue, 1974). Upon germination, a large increase in LOX activity was noted after 3.5 days (Guss et al, 1968a). The activity was mainly localized in the coleoptile plus the first leaf. On a dry-weight basis, the root was the next most active tissue. After seven days of germination, the activity decreased throughout the seedling, but the main site of LOX remained in the coleoptile plus the first leaf.

PROPERTIES OF CRUDE LOX ENZYMES
Although crude extracts of wheat are now known to be mixtures of LOX isoenzymes, a number of investigators have characterized the properties of these mixtures. Compared to oxidation activity of linoleic acid (100%), the preferred substrate of wheat LOXs, the activity of methyl linoleate was only 10% and of trilinolein; dilinolein, and monolinolein about 3% (Guss et al, 1968b). Digalactosyldiglyceride was not a substrate. The pH optimum of wheat LOXs has been variously reported at 5.7 (Kieffer et al, 1982), 4.8 (McDonald, 1979), 5.9 (Walsh et al, 1970), and 6.5 (Irvine and Anderson, 1953a). The latter workers reported a $K_m$ of $5.0 \times 10^{-6} M$ and an activation energy of 6.5 kcal. Siddiqi and Tappel (1957) determined that wheat LOXs were inhibited by nordihydroguaiaretic acid, propyl gallate, $\alpha$-tocopherol, and hydroquinone, in descending order of potency. Competitive inhibition of wheat LOXs was obtained with L-ascorbic acid (Walsh et al, 1970). Inhibition was obtained with CNF only, provided that the extract was preincubated before addition of substrate (Irvine and Anderson, 1955). It is remarkable that this early indication of the involvement of iron in LOX was not pursued further.

A number of investigators have observed the interaction of wheat LOXs with proteins. According to Nicolas et al (1981a, 1981b), wheat flour contains an inhibitor of wheat LOXs, probably a protein, that acted on linoleic acid rather than on the LOXs. Casein inhibited wheat LOXs, as well as the LOXs from rye, oats, and barley (Laakso and Lilius, 1982). The inhibition was not due to the presence of $Ca^{2+}$ in casein nor to substrate absorption by casein. In wheat flour, approximately 50-70% of the LOX activity was bound to gluten (Kretovich et al, 1980), and the bound activity was released by reduced glutathione (Ikhsanov et al, 1983). As discussed below, the gluten-binding properties of LOX isoenzymes purified to homogeneity or near homogeneity have been studied in detail.

Wheat LOXs were relatively easily inactivated. When a wheat protein concentrate was stored at 3°C with moistures greater than 13.1%, the LOXs
were unstable, but at \(-18^\circ C\) or colder, the LOXs were stable at any moisture (Wallace and Wheeler, 1972). At 24°C the LOXs in aqueous suspension were 50% inactivated within 2 hr, and nearly complete inactivation was achieved with a 30-min heat treatment at 65°C and 16.3% moisture. Like LOX from soy (Smith and Lands, 1972), the wheat LOXs catalyze their own destruction during the oxidation of substrate (Wheeler and Wallace, 1973a).

COOXIDATION BY CRUDE LOX ENZYMES

As early as 1953, Blain et al studied the bleaching of carotene by cooxidation reactions caused by the action of wheat LOXs on substrate fatty acids. Compared with the LOX-2 and LOX-3 isoenzymes from soybeans, a partially purified preparation of wheat LOXs was a relatively poor cooxidizer of carotene (Grosch et al 1976). According to the latter workers, the soy LOX-2 and LOX-3 were more efficient cooxidizers because they tended to release more free radicals from the active site. Characteristics that were indicators of cooxidation potential were large yields of volatile secondary products and nonspecific positional oxidation of the fatty acid substrate, neither of which described the properties of their wheat LOX preparation. Wheat LOX activity was highly correlated with the extent of lutein bleaching (McDonald, 1979); however, bleaching occurred at a different pH optimum (pH 9.0) than LOX activity (pH 4.8). Ascorbic acid inhibited lutein bleaching more effectively than it inhibited LOX oxidation of fatty acids, indicating that a quenching of free radicals by ascorbic acid may have blocked cooxidation. Chlorophyll bleaching activity in wheat leaf homogenates has been attributed to LOX (Imamura and Shimizu, 1974). The activities of LOX and chlorophyll bleaching were inseparable by gel-filtration on a Sephadex G-200 column.

OXIDATION SPECIFICITY OF CRUDE LOX ENZYMES

The positional specificity of substrate oxidation by crude wheat LOXs has an inherent degree of uncertainty because the individual isoenzymes may differ in this regard. Graveland (1970a) reported that LOXs in flour-water suspensions or doughs oxidized linoleic acid into an 85:15 ratio of 13-LOOH to 9-LOOH. Later Graveland et al (1972) and Graveland (1973a) reported that the actual composition of the oxidation mixture obtained by these preparations was the reverse, that is, a 15:85 ratio of 13-LOOH to 9-LOOH. The oxidation of linolenic acid by flour-water suspensions resulted in only secondary products, but the structure of these products, such as 9-hydroxy-10,12,15-octadecatrienoic acid, indicated a specific oxidation to the 9-hydroperoxide of linolenic acid (9-LnOOH) (Graveland, 1973b). Using partially purified wheat LOXs to oxidize linolenic acid, Grosch et al (1976) also found a predominant specificity for carbon-9; they found a 9-LnOOH to 13-LnOOH ratio of 85:15.

SEPARATION OF ISOENZYMES

Guss et al (1967) first detected the existence of four LOX isoenzymes in wheat by electrophoretic separation on polyacrylamide gel. Their communication was followed by others from the same laboratory (Guss et al, 1968a, 1968b; Hale et al, 1969). Guss et al (1968a), who examined LOXs from germinated seedlings, showed that the intensity of the isoenzyme bands separated on the gels varied according to the tissue from which the isoenzymes were isolated as well as the
stage of tissue development. Similar findings were reported by Oganesyan et al (1983).

After the discovery of LOX isoenzymes, a number of laboratories attempted their isolation. Unfortunately, each group of workers used a different procedure and numbering system for the isoenzymes; therefore, data comparison is difficult. Graveland (1970b) separated two isoenzymes from wheat flour by sequential passage through Sephadex G-100 and carboxymethyl (CM)-Sephadex columns. Two major and two minor LOX isoenzymes were obtained from defatted whole wheat by precipitation with \((\text{NH}_4)_2\text{SO}_4\) (30–60\% of saturation) followed by separation with a CM-Sephadex column (Weber et al, 1973). Wallace and Wheeler (1975) separated four isoenzymes from defatted wheat germ using a three-step isolation procedure of precipitation with \((\text{NH}_4)_2\text{SO}_4\) (35–55\% of saturation), followed by gel filtration with a Sephadex G-200 column and elution through a diethylaminoethyl (DEAE)-cellulose column. Subsequent work by Wallace and Wheeler (1979) indicated that only two isoenzymes were present in wheat germ and that the two additional isoenzymes were artifacts derived from one of the two native forms. They isolated the two isoenzymes by chromatography on a CM-cellulose column after fractional solubilization from \((\text{NH}_4)_2\text{SO}_4\)-precipitated protein. Each of the two was subjected to further chromatographic separation on either DEAE-cellulose or CM-cellulose columns. LOX-1, which was purified 40,000-fold, was not stable above pH 7, and it degraded into two additional active LOXs. The other isoenzyme, LOX-2 (purified 1,200-fold), did not degrade into other active forms. Nicolas et al (1982) obtained three isoenzymes from defatted wheat germ by a four-stage procedure: extraction at pH 4.5, precipitation with \((\text{NH}_4)_2\text{SO}_4\) (25–40\% of saturation), gel filtration with Ultrogel AcA34, and chromatography on DEAE Sephadex. Since these investigators used pH 7.0 buffer during part of the procedure, it is conceivable that one of their isoenzymes was an artifact, as described by Wallace and Wheeler (1979). Three LOX isoenzymes were recovered from durum wheat flour by extraction at pH 5.0, fractional precipitation with \((\text{NH}_4)_2\text{SO}_4\) (30–60\% of saturation), and sequential chromatography on DEAE-cellulose and CM-cellulose columns (Hsieh and McDonald, 1984). Since the order of isoenzyme elution was labeled in reverse by Wallace and Wheeler (1979), Hsieh and McDonald (1984) pointed out that their LOX-2 and LOX-3 may correspond to LOX-2 and LOX-1, respectively, of Wallace and Wheeler (1979). The other isoenzyme (LOX-1) isolated by Hsieh and McDonald (1984) possessed LOX, peroxidase, and carotene-bleaching activities. Kühn et al (1985) purified an apparent isoenzyme from wheat caryopses by fractional \((\text{NH}_4)_2\text{SO}_4\) precipitation, gel filtration on Sephadex G-200, and isoelectric focusing.

No rigorous proof of isoenzyme purity was done for any of the wheat LOXs described above. However, gel electrophoretic patterns were either illustrated or described in the investigations of Wallace and Wheeler (1979), Nicolas et al (1982), and Hsieh and McDonald (1984). Except for some minor impurities, most of these isoenzymes appeared to have been isolated. Nicolas et al (1982) claimed to have isolated two isoenzymes on the basis of the purity of their electrophoretic bands. The degree of purification claimed by these three investigators ranged over orders of magnitude. Purification from wheat flour ranged from 16- to 28-fold and from wheat germ 201- to 40,000-fold.
PROPERTIES OF PURIFIED ISOENZYMES

The molecular weights of wheat LOX isoenzymes were close to those observed for the soybean LOXs. Wallace and Wheeler's (1979) two isoenzymes ranged in size from 84,000 to 89,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis. For their three isoenzymes, Nicolas et al (1982) reported a range of 90,000-95,000 by gel filtration and 110,000 by electrophoresis on a polyacrylamide gel concentration gradient.

The pH optima of most isoenzymes from wheat were near neutrality. According to Wallace and Wheeler (1975, 1979) and Hsieh and McDonald (1984), a number of isoenzymes shifted in pH optima as a result of changes in substrate concentration. The ranges in pH optima reported for various isoenzymes from wheat germ were 6–7 (Wallace and Wheeler, 1979), 6–6.5 (Nicolas et al, 1982), and 4–7 (Wallace and Wheeler, 1975). The three wheat flour isoenzymes displayed a substantial difference in pH optima, 4.8 for two and 10.2 and 11.4 for the third (Hsieh and McDonald, 1984).

Nicolas et al (1982) were the only investigators to report pI values for wheat LOX isoenzymes. By isoelectric focusing they determined pI values of 5.1, 5.4, and 6.25 for their three isoenzymes.

Enzyme inhibitors have been tested with wheat isoenzymes. Two isoenzymes were partially inactivated by sodium azide, indicating an iron-active site (Wallace and Wheeler, 1979). The latter workers showed that the response to thiol reagents was variable. One isoenzyme was stable after treatment with p-chloromercuriphenylsulfonic acid and N-ethylmaleimide, while the other was completely inactivated. They also found that both isoenzymes were partially inactivated by a thiol, dithiothreitol. This behavior was reminiscent of the inactivation of soybean LOX by thiols (Roza and Francke, 1973). The wheat isoenzymes also were inhibited by excess substrate concentration (Wallace and Wheeler, 1975). As a consequence, a double reciprocal plot of velocity versus substrate concentration was a sigmoidal curve (Wallace and Wheeler, 1979).

According to Wallace and Wheeler (1979), one isoenzyme had a kinetic lag phase, which was overcome by product hydroperoxide, but the other did not have an observable lag.

Nicolas (1982) reported that the wheat isoenzymes lose about 50% of their activity when stored at 4° C for two weeks to a month.

ISOENZYME BINDING TO GLUTEN AND HYDROPHOBIC SURFACES

Apparently, in wheat flour-water mixtures or doughs, LOX becomes bound to gluten. Glutenin, rather than gliadin, was the component of gluten that appeared to bind the LOX, and this adsorption was reversible (Graveland, 1970b). As LOX was further purified, the maximum adsorption of the enzyme became greater. The adsorption isotherm was described by a Langmuir equation. Heat treatment or reduction by NaBH₄ released the LOX, but the NaBH₄ treatment could be reversed by air oxidation. The reversal by air oxidation was prevented by blocking thiol groups with N-ethylmaleimide. Adsorption was also reduced after solvent extraction of the gluten, the more polar solvents being more effective. Whereas the nonbound LOX formed mainly hydroperoxides from the oxidation of linoleic acid, the bound LOX produced mainly the secondary product, THA. The formation of trihydroxy fatty acids is
Wheeler and Wallace (1973b) described the binding of wheat LOX isoenzymes to hydrophobic surfaces, much like the adsorption to gluten described above. During assays for activity, they noted that LOX became bound to the teflon surface of the O₂ electrode and also to the epoxy-coated stirring bars used in the assays. The binding became more pronounced as the enzyme was further purified. Addition of substrate, linoleic acid, or product hydroperoxide to LOX before exposure to the hydrophobic surface prevented the binding. The approximate Kₘ for linoleic acid with the free LOX was 5 × 10⁻⁶ M, while the value for the bound LOX was 100-fold higher.

OXIDATION SPECIFICITY OF ISOENZYMES

Since wheat contains more than one LOX isoenzyme, it is obviously important to test the oxidation specificity of each isoenzyme individually. A pure isoenzyme from wheat caryopses oxidized linoleic acid to better than 91% 9-LOOH (Kühn et al., 1985). This oxidation specificity was typical of LOX isoenzymes from quiescent cereal seeds but atypical of those from soybeans. The wheat enzyme also differed from soy LOXs in the oxidation of arachidonic acid. The wheat LOX oxidized arachidonic acid toward the carboxylic acid end of the fatty chain, affording a 67:33 ratio of the 8- and 5-hydroperoxides (Kühn et al., 1985), whereas soy LOX oxidized the ω end to give the 15-hydroperoxide (Hamberg and Samuelsson, 1967). Kühn et al. (1985) found that only those fatty acids with a double-allylic methylene carbon localized at C-10 and C-11 were oxidized by wheat LOX. By contrast, soybean LOX requires an ω8 or ω4 double-allylic methylene carbon. That is, the wheat enzyme used the carboxylic acid group of the fatty acid as a recognition site for oxidation, rather than the methyl end used by the soybean LOX. Kühn et al. (1985) further demonstrated a dependence on the carboxylic acid group by showing that chemical modification of the carboxylic acid either greatly reduced or terminated oxidation.

An interesting feature of the oxidation of linoleic acid by wheat LOX isoenzymes was the shortfall in O₂ absorption for a given quantity of substrate (Wallace and Wheeler, 1975). The computed ratio of O₂ to linoleic acid was 0.7, yet ultraviolet absorbance at 234 nm indicated that conjugated hydroperoxydiene products were quantitatively formed. Apparently the reaction is partly diverted into products other than LOOH that also absorb ultraviolet at 234 nm. The formation of conjugated octadecadienoic acid dimers by anaerobic-type reactions (Figure 3) did not seem likely because the other anaerobic product, oxooctadecadienoic acid, appeared to be absent.

COOXIDATION BY ISOENZYMES

The association of carotene-bleaching activity with LOX isoenzymes in wheat appears to be universal. The peaks of carotene-bleaching activity comigrated exactly with the peaks of all the LOX isoenzymes separated from either whole wheat (Weber et al., 1973), wheat germ (Nicolas et al., 1982), or wheat flour (Hsieh and McDonald, 1984). In addition to LOX and carotene-bleaching activities, one isoenzyme also displayed peroxidase activity (Weber et al. 1973; Hsieh and McDonald, 1984). Hsieh and McDonald (1984) showed that the peroxidase activity could not be separated from either LOX or carotene-bleaching activities even after affinity chromatography by a concanavalin
A-type column. With the three flour isoenzymes, the pH optima for the LOX activity was lower than for the carotene-bleaching activity, except for one (Hsieh and McDonald, 1984).

In studies with soybeans and other plants, carotene-bleaching and LOX activities were always highly correlated to such an extent that they were assumed to be identical, and carotene bleaching always required LOX substrates. The data with wheat again strongly indicated that carotene-bleaching and LOX activities were the same.

D. LOX in Bread and Pasta

OVERVIEW AND HISTORICAL PERSPECTIVE

LOX has a multifaceted effect on bread-baking. In doughs, the enzyme has been implicated in bleaching of carotenoids, improvement of rheology, release of bound lipid, and increased mixing tolerance. Carotenoid bleaching affects also pasta in that it is detrimental to the retention of the bright yellow color of pasta. The role of LOX (and O₂) in bread-baking has been reviewed by Hawthorn (1961), Bloksma (1964), Daniels et al (1971), Eskin et al (1977), Morrison (1978), Frazier (1979), Faubion and Hoseney (1981), Nicolas and Drapron (1983a, 1983b), and Galliard (1983).

An improver function for LOX in breadmaking has been recognized for more than 50 years. Soy flour, containing LOX, was used in bread batters by Haas and Bohn (1934) to bleach wheat carotenoids in the production of white bread. Subsequently, Sullivan et al (1936) surmised that "oxidases" indigenous to wheat flour were responsible for the oxidation of unsaturated fatty acids to peroxides during dough mixing. According to them, this resulted in tough, short glutens. Two decades later research demonstrated that flour doughs consumed O₂ (Hawthorn and Todd, 1955; Cosgrove, 1956; Smith and Andrews, 1957). Because O₂ uptake was diminished by fat extraction of the flour, restored by addition of linoleic acid, and terminated by heating, the action of LOX was suggested (Smith and Andrews, 1957).

LOX OXIDATION OF DOUGH LIPIDS

After LOX activity was implicated in the mixing of doughs, a number of investigations established that lipids were indeed oxidized. Accumulation of Lipid hydroperoxides during dough mixing was measured by the thiobarbituric acid test (Tsen and Hlynka, 1962). Morrison (1963) and Morrison and Maneely (1969) suggested that LOX activity only accounted for a portion of the O₂ consumed by flour-water suspensions and that saturated fatty acids and oleic acid were also oxidized; however, subsequent work by the same laboratory showed that only the polyunsaturated fatty acids were being oxidized (Mann and Morrison, 1974). According to Graveland (1968) and Mann and Morrison (1974), the in situ substrates for flour LOX were the free fatty acids, linoleic and linolenic acids, and α-monoglycerides containing a polyunsaturated fatty acid residue. The products of oxidation were hydroperoxides of either free fatty acids or α-monoglyceride fatty acid. Fatty acid hydroperoxides were converted mainly into the secondary products, hydroxy-, epoxyhydroxy-, and trihydroxy-fatty acids (Graveland, 1970a, 1970b, 1973a, 1973b, 1973c; Mann and Morrison, 1975). Secondary products are discussed in more detail in the section on reactions of hydroperoxides.
When soy flour supplements are added to wheat doughs, a complete change in the character of the oxidation usually occurs. Because soy is a rich source of LOX, increased oxidation is possible. Furthermore, soybean contains a LOX isoenzyme, LOX-2, that oxidizes triglycerides, and both soy LOX-2 and LOX-3 are efficient cooxidizers. The addition of soy to doughs resulted in losses of polyunsaturated fatty acids from the steryl esters, triglycerides, diglycerides, galactolipids, and phosphatidylyceroline (Morrison and Panpaprai, 1975). All classes of lipids were peroxidized in soy-supplemented doughs (Daniels et al., 1970). and soy was found to cause a drop in the linoleic acid content of dough triglycerides (Daniels et al., 1968).

CAROTENOID BLEACHING

The bleaching of carotenoids in bread by LOX has been reviewed by Faubion and Hoseney (1981). Although LOX(s) indigenous to wheat flour were capable of partially bleaching carotenoids in doughs, supplementation with soy flour resulted in more efficient bleaching (Haas and Bohn, 1934; Mann and Morrison, 1975). As discussed in the previous section, the soy isoenzymes, LOX-2 and LOX-3, are undoubtedly responsible for efficient bleaching action. These two isoenzymes are particularly well known for their cooxidation potential. In addition to soy flour, broad bean (Vicia faba) flour, has been used to bleach bread (Chargelegue, 1974; Drapron et al., 1974). Intensified kneading caused more efficient cooxidation of the carotenoids, as well as α-tocopherol, in the broad bean-amended doughs (Drapron et al., 1971, 1974; Drapron, 1973). In these amended doughs, the antioxidant, butylated hydroxytoluene, and the LOX inhibitors, ascorbic acid and H₂O₂, decreased carotenoid destruction (Nicolas et al., 1978).

Although LOX has been used in bread baking to bleach carotenoids, the enzyme is deleterious in pasta manufacture because the bright yellow color of carotenoid is a pasta quality indicator. Adding linoleic acid to pasta dough and mixing with O₂ resulted in considerable carotenoid loss, whereas lack of substrate or the heat inactivation of LOX prevented pigment loss (Matsuo et al., 1970). Mixing pasta dough either with ascorbic acid (Walsh et al., 1970) or in a vacuum (Burov et al., 1974) also prevented carotenoid losses. According to Walsh et al. (1970), ascorbic acid was a competitive inhibitor of wheat LOX. Laignelet (1983) showed that the water-soluble fraction of durum wheat semolina was primarily responsible for carotenoid bleaching in pasta.

LOX INTERACTION WITH GLUTEN: THIOL OXIDATION AND LIPID BINDING

LOX oxidation of lipids results in a number of changes in doughs. Most important to bread-baking is the improved dough rheology, which results in larger loaf volumes and improved texture. Also, the ability of dough to resist breakdown from overmixing after reaching peak development is increased by the action of LOX.

The mechanisms by which this enzyme improves dough rheology and mixing tolerance are subject to different interpretations. The most accepted theory holds that the oxidation of thiol groups in gluten is responsible. Some investigators believe the release of bound lipid caused by LOX action is additionally important.
Historically, oxidizing agents, such as bromate and iodate, have been known to improve the rheological properties of wheat doughs. Oxidizing agents are believed to convert sulfhydryl groups of gluten protein into disulfide bonds. The deformation of dough probably involves the breaking and reformation of disulfide bonds through sulfhydryl-disulfide interchange (Bloksma, 1964). Any loss in this interchange due to the unavailability of sulfhydryl groups should lead to rigidity. Consequently, one might expect that lipid hydroperoxides or the active formation of radicals from LOX action might also decrease the sulfhydryl groups in bread doughs. Lipid hydroperoxides oxidize cysteine into cystine in the presence of iron ions (Lewis and Wills, 1962).

Early workers (Koch, 1956; Smith et al, 1957; Tsen and Hlynka, 1962, 1963) showed that the oxidation of polyunsaturated fatty acids by LOX led to oxidation of sulfhydryl groups in doughs. Tsen and Hlynka (1962, 1963) developed the important concept that sulfhydryl groups were directly oxidized by O₂, as well as by lipid peroxidation. Thus, O₂ could react both with sulfhydryl groups directly and with unsaturated lipids via LOX action. Subsequently, lipid hydroperoxides from LOX activity could further oxidize sulfhydryl groups. Thus, it could be surmised that under certain conditions lipids and sulfhydryl groups could compete for available O₂. Dahle and Sullivan (1963) concluded that the role of lipid peroxidation in oxidizing sulfhydryl in doughs was relatively minor, and Sullivan et al (1963) reported that only about half the sulfhydryl groups, mainly water-soluble sulfhydryls, were lost during dough mixing. Graveland et al (1976) claimed that normal wheat flour contained 5-7 μmol of thiol per gram of flour, of which 0.5-1.5 μmol per gram became oxidized by dough mixing. According to Bloksma (1964), the conversion of a relatively few sulfhydryls into disulfide groups could significantly reduce the rate of interchange reactions. This could explain how the loss of only a portion of the sulfhydryl groups could cause a considerable increase in a dough’s resistance to deformation. More recently, Sidhu et al (1980) demonstrated the involvement of thyl radicals of cysteine during the mixing of dough. They mixed ¹⁴C-fumaric acid with dough and showed that the label was incorporated into protein as S-succinylcysteine. A well known reaction of thyl radicals is their addition to double bonds, such as was observed with fumaric acid. Supplementation with soy flour results in considerable improvement in dough rheology, and it is not surprising, therefore, that the addition of soy flour led to the loss of more sulfhydryl groups (Auerman et al, 1971b). Despite the fact that the sulfhydryl-disulfide interchange theory is the best model yet advanced to explain the oxidative changes in dough rheology, Faubion and Hoseney (1981) stated that the interchange theory of dough improvement is a “severe overgeneralization” and that “no model yet exists to explain the effect of LOX on wheat flour rheology.”

The release of bound lipid from dough by LOX activity is an interesting phenomenon, which was reviewed by Daniels et al (1971) and Frazier (1979). Because the improvement of dough rheology correlates with lipid release, Daniels et al (1971) and Frazier et al (1973) believe lipid release is directly involved in rheology. However, no tangible evidence exists to show a causal relationship. Both Frazier (1979) and Daniels et al (1971) discussed the “fat failure” encountered during extreme lipid binding, such as observed during the working of dough under N₂. These workers maintain that free lipid must be
available to benefit both proof and oven performance. According to Frazier et al (1977), the presence of free lipid in dough assists in the relaxation of internal stress during baking.

Olcott and Mecham (1947) first observed that lipids were adsorbed to the glutenin fraction of dough gluten. The binding was largely physical, and lipid could be extracted by polar solvents, such as CHCl₃-CH₃OH-H₂O or butanol-H₂O. If doughs were mixed under N₂, the lipid binding continued to increase during mixing (Daniels et al, 1967). Under N₂, the increase in lipid binding was dependent on both the work rate and total work level of mixing. The introduction of air into the dough released the bound lipids, and the decreased binding was dependent only on the total work input. According to Daniels et al (1966), triglyceride was the predominant bound lipid, possibly because it was the major lipid present in dough. Except for the polar lipids, no preference for unsaturation was observed by them. As observed by Daniels et al (1971), the release of lipid by air-mixing was due to LOX activity. They additionally found that lipid release during air-mixing was dependent on supplying both LOX and the proper substrate for the particular source of LOX. As expected, indigenous wheat flour LOXs, soy flour LOXs, and purified soy LOX had different substrate requirements to release lipid from dough.

Like dough lipids, LOX also binds to the glutenin fraction of dough gluten (Graveland, 1970b). Furthermore, the extraction of lipid from gluten inhibited the adsorption of LOX. Graveland (1970b) also noted that LOX adsorption was prevented by disulfide reduction and subsequent derivatization of the sulfhydryls. It is tempting to speculate that such an intimate interaction between lipid, LOX, and glutenin plays a significant role in the physical properties of doughs.

Daniels et al (1970) and Frazier (1979) theorized that a coupled LOX oxidation of sulfhydryl groups was responsible for release of lipid from binding. The primary evidence for coupled oxidation concerns the fact that addition of preperoxidized lipid to dough did not release bound lipid during mixing under N₂ (Daniels et al, 1970, 1971). Thus, the active oxidation of lipid by LOX affords a reactive intermediate and/or free radicals, and these, rather than the direct action of lipid hydroperoxides themselves, cause the observed changes in lipid binding.

DOUGH RHEOLOGY

As discussed above, the principal practical reason for the interest in LOX action in bread-baking relates to the improvement in dough rheology. Dough rheology can be measured either by increased relaxation times as a function of work input by kneading (Frazier, 1979) or by the rate of spread of a molded dough piece (Faubion and Hoseney, 1981). Such measurements should predict the proper consistency of dough gluten, which leads to retention of gas and increased loaf volume in baking.

As early as 1936, Sullivan et al noted the formation of short, tough gluten from the addition of unsaturated fatty acids to dough. They correctly hypothesized the involvement of fatty acid oxidases. Early workers also showed that supplementation with less than 1% soy flour significantly increased gluten strength (Logan and Learmonth, 1955). At the 5% level, soy was detrimental (Ofelt et al, 1954), possibly because of soy proteases (Ofelt et al, 1955). The
rheological data indicative of improvement with LOX activity were confused when research demonstrated that the rheology of doughs was improved with defatted flour (Smith et al., 1957; Narayanan and Hlynka, 1962). Air-mixing dough prepared from defatted flour resulted in slightly improved loaf volumes compared to the same dough mixed under N₂ (Hawthorn and Todd, 1955). Perhaps these observations can be explained by the complexities of sulfhydryl oxidation (Tsen and Hlynka, 1962, 1963), that is, lipids and sulfhydryl groups can be competitive for the consumption of O₂. As noted by Frazier (1979), variations in the availability of O₂ under different conditions may explain the apparent improvement in dough rheology with defatted flour. Considering the complexity of the ingredients in dough, it is doubtful that the rheology of defatted flour dough is completely understood. For example, Bloksma (1963) reported that fat extraction does not result in increased oxidation of sulfhydryls in dough.

Although endogenous wheat flour LOX has been implicated in the improvement of dough rheology, no correlation was obtained between LOX activity and baking performance of doughs made from the flour of 11 different wheat varieties (Kieffer et al., 1982). As discovered by early workers (Logan and Learmonth, 1955), the addition of less than 1% LOX-rich soy flour causes improvement of dough rheology, and this has been confirmed by many investigators (Daniels et al., 1966, 1968, 1970, 1971; Auerman et al., 1971b; Frazier et al., 1973, 1977, 1979; Morrison and Panpaprai, 1975; Frazier, 1979; Hoseney et al., 1980). The increase in loaf volume due to soy supplementation can be striking (Frazier, 1979). The considerable improver action of soybean flour has been attributed to its content of LOX-2, which, unlike wheat LOX, is capable of oxidizing triglyceride polyunsaturated fatty acid (Mann and Morrison, 1975). Grosch and Kieffer (1980) and Kieffer and Grosch (1980) have demonstrated that isolated soy LOX-2 can replace crude soybean extracts to improve baking performance. Additionally, soy LOX-2 is relatively effective in cooxidation reactions, which may have a bearing on dough improvement, as discussed later in this section.

Besides soy, other sources of LOX have been used in bread-baking, such as broad bean (Chargelegue, 1974; Etienne and Dubois, 1974), navy bean (D’Appolonia, 1978), and wheat protein concentrate (Wallace and Wheeler, 1972). Navy bean and wheat protein concentrate decreased baking performance unless they were first inactivated by heat. The heat improvement of navy bean was probably due to inactivation of a protease.

According to Frazier et al. (1977), nordihydroguaiaretic acid greatly inhibited hydroperoxide formation by LOX but only marginally impaired rheological improvement of doughs. According to them, the improver effect on dough was due to a coupled oxidation requiring active oxygenation of a lipid substrate by LOX, rather than by the product of LOX action. In this regard, the requirement for coupled oxidation seemed to parallel similar observations with lipid binding and carotenoid bleaching. As mentioned above, soy LOX-2, which is known to be an effective cooxidizing isoenzyme, was shown to improve dough rheology (Kieffer and Grosch, 1980). It does seem plausible that the active cycling of free radical intermediates would be much more effective in producing oxidative changes in protein than would the lipid hydroperoxides themselves.

In addition to nordihydroguaiaretic acid, the effects of a number of
Lipoxygenase Pathway in Cereals

Antioxidants on dough rheology have been studied. These results have been variable, making interpretation difficult. For example, ascorbic acid either did not affect dough (Dahle and Murthy, 1970) or improved dough rheology and loaf volume (Meuser et al., 1978; Frazier et al., 1979). Note that ascorbic acid can display dual antioxidant/prooxidant effects. In the presence of transition metal ions, ascorbic acid can reduce the metal ions, which in turn lead to radical degradation of lipid hydroperoxides. On the one hand, the antioxidants tocopherol, propyl gallate, nordihydroguaiaretic acid, butylated hydroxytoluene, and butylated hydroxyanisole improved dough rheology (Narayanan and Hlynka, 1962), but propyl gallate (Auerman et al., 1971c), hydroquinone, and p-benzoquinone (Dahle and Murthy, 1970) caused deterioration of rheological properties.

Mixing Tolerance

Mixing tolerance concerns the ability of dough to resist breakdown from overmixing after reaching peak development. This characteristic is usually evaluated by the retention of increased dough relaxation times with increased levels of work input using a mixograph. Mechanistically, mixing tolerance and dough rheology are possibly related, but they are evaluated separately as important characteristics in bread-baking technology. According to Frazier et al. (1973, 1977), supplementation of doughs with soy flour resulted in the maintenance of increased relaxation times with higher work input (kneading) before dough breakdown. Because increased mixing tolerance only occurred in the presence of polyunsaturated fatty acid substrate (Frazier et al., 1977; Hoseney et al., 1980) and O₂ (Frazier et al., 1977), LOX was implicated. Heat-denatured soy flour did not increase mixing tolerance (Frazier et al., 1973).

Hoseney et al. (1980) showed that a soy supplement could overcome the deleterious effect of KIO₃ on mixing tolerance. This effect was demonstrated even under an N₂ atmosphere.

Retardation of Staling

An unexpected result of soy supplementation was a marked retardation of bread staling (Frazier, 1979). Staling is usually considered to be the result of starch retrogradation, but the effect of soy seems to indicate that the gluten component of bread is involved.

Off-Flavors

Flavor problems are often encountered after supplementation with various bean flours (Chargelegue, 1974; D'Appolonia, 1978), especially after intensified kneading (Étienne and Dubois, 1974). With broad bean supplementation, increased amounts of hexanal presumably arise from the decomposition of 13-LOOH originating from LOX activity (Drapron and Beaux, 1969; Drapron, 1973; Drapron et al., 1974). Pentanol, hexanal, hexanol, trans-2-octenal, trans-2-nonenal, and 2,4-decadienal were among the volatiles detected by Heimann et al. (1979) in pastry that had been supplemented with soy flour. These volatiles were also detected after the addition of purified LOX to the pastry dough. The production of volatiles as a secondary reaction of LOX has been reviewed recently (Gardner, 1985a).
E. Barley LOX

HISTORICAL PERSPECTIVE

Apparently, Kolesnikov (1950) first reported LOX in barley. Some properties of the crude enzyme were characterized a few years later by Franke and Frehse (1953).

DISTRIBUTION IN THE SEED

In barley, 98-99% of the LOX activity resides in the germ (Lulai and Baker, 1976; Lulai et al, 1981). Germination of the seed greatly stimulated LOX activity in the embryo but did not elicit activity in the endosperm (Yabuuchi and Amaha, 1975; Lulai et al, 1981). The increase in LOX activity during germination was confirmed by von Ceumern and Hartfiel (1982) and Schwarz and Pyler (1984). According to Lulai and Baker (1975), the distribution of activity in the germinated seed was 49% in germ, 27% in acrospire, and 24% in rootlets.

SEPARATION OF ISOENZYMES

Polyacrylamide gel electrophoresis afforded only one LOX isoenzyme band from quiescent barley seeds (Lulai and Baker, 1976). Yabuuchi and Amaha (1975) also concluded that only one isoenzyme was present in the ungerminated seed. They purified this isoenzyme 11-fold by fractional precipitation with (NH₄)₂SO₄, followed by gel filtration twice through a Sephadex G-150 column and once through a Sephadex G-200 column. Heimann and Timm (1977a) also partially purified the barley seed LOX by (NH₄)₂SO₄ fractionation and gel filtration with Sephadex G-150. Isolation of the seed isoenzyme was achieved by Führling (1975), who obtained a 137-fold purification. For isolation, he utilized (NH₄)₂SO₄ precipitation and sequential passage through columns of Biogel A-0.5 m, SP-Sephadex C-50, and Sephadex G-150.

After seed germination, a second isoenzyme (LOX-2) appeared, which increased from the third to the seventh day of germination (Yabuuchi, 1976). LOX-2 was more unstable and resisted purification. However, a 30-fold purification was obtained by chromatography with DEAE Sephadex. Thus, Yabuuchi (1976) designated the isoenzyme from quiescent barley seed as LOX-1, and this isoenzyme presumably is identical to the one characterized by Lulai and Baker (1976), Führling (1975), and Yabuuchi and Amaha (1975).

PROPERTIES OF ISOENZYMES

The molecular weight of LOX-1 was determined by gel filtration to be 64,000 (Führling, 1975), and its $K_m$ was reported to be $10^{-3} M$ by Führling (1975) and 2.6 $\times 10^{-4} M$ by Lulai and Baker (1976). The activation energy of LOX-1 was set at 2.2 kcal/moi by the latter workers. The pH optimum values of LOX-1 were determined to be 5.9–6.0 (Lulai and Baker, 1976), 7.8 (Heimann and Timm, 1977a), 6.5 (Führling, 1975), and 7.5 (Yabuuchi and Amaha, 1975; Yabuuchi, 1976). According to Yabuuchi (1976), the pH optimum of LOX-2 was 7.0–7.5. The pH values for LOX-1, reported at 4.75 (Führling, 1975) and 4.9 (Yabuuchi, 1976), differed considerably from the pH 6.6 of LOX-2 (Yabuuchi, 1976).

LOX-1 was resistant to freezing and freeze-drying (Yabuuchi and Amaha, 1975), and was reasonably stable up to 50°C (Lulai and Baker, 1976). According to Yabuuchi (1976), LOX-2 was much more unstable.
A number of reagents were tested with LOX-1 for possible inhibition. No inhibition was obtained with CN⁻ and ethylenediamine tetraacetic acid (EDTA) (Yabuuchi and Amaha, 1975; Lulai and Baker, 1976), but these workers disagreed on the effects of Hg²⁺ and Cu²⁺. Unlike the latter two investigators, Führling (1975) noted partial inhibition with CN⁻. According to Lulai and Baker (1976), the sulfhydryl reagents, p-chloromercuribenzoate and N-ethylmaleimide, inactivated LOX-1, but dithiothreitol and cysteine did not. Additionally, LOX-1 was inhibited by nordihydroguaiaretic acid, H₂O₂, iodoacetamide (Führling, 1975), ascorbic acid (Lulai and Baker, 1976), and the product of its reaction, linoleic acid hydroperoxide (Yabuuchi and Amaha, 1975). Contrary to the findings of Franke and Frehse (1953), no activation was obtained with Ca²⁺ (Yabuuchi and Amaha, 1975; Lulai and Baker, 1976).

LOX-1 and LOX-2 also differed in their preference for substrate. Linolenic acid (Lulai and Baker, 1976) and linoleic acid were substrates for LOX-1, but trilinolein and methyl linoleate were not (Yabuuchi and Amaha, 1975; Lulai and Baker, 1976; Yabuuchi, 1976; Heimann and Timm, 1977a). LOX-2 readily oxidized linoleic acid, trilinolein, and methyl linoleate (Yabuuchi, 1976).

**OXIDATION SPECIFICITY OF ISOENZYMES**

Barley LOX-1 favored oxidation of linoleic acid predominantly at carbon-9, with minor oxidation at carbon-13. The reported percentages of 9-LOOH were 70% (Lulai et al., 1981), 89% (Graveland et al., 1972), 90% (Yabuuchi and Amaha, 1975; Heimann and Timm, 1977a), and 96% (Führling, 1975). Heimann and Timm (1977a) noted a shift in percentage of 9-LOOH from 90% at pH 7 to 70% at pH 7.75, which was similar in direction to the pH dependence of oxidation specificity noted for soybean LOX-1.

Yabuuchi (1976) showed that linoleic acid was oxidized by barley LOX-2 to 90% 13-LOOH, with the remainder being the 9-LOOH isomer. Since LOX-2 appeared after germination, it is not unusual that Lulai et al. (1981) noted an increase in the production of 13-LOOH by a barley extract prepared from germinated seeds. Because of the oxidation specificity for carbon-13, Yabuuchi (1976) postulated that the green-note aroma (hexanal/hexenals) of malted barley originated from the action of the LOX-2 isoenzyme.

**F. Maize LOX**

**HISTORICAL PERSPECTIVE**

Franke and Frehse (1953) and Fritz and Beevers (1955) were the first to study LOX activity in maize. Wagenknecht (1959) attributed the development of off-flavor in frozen corn-on-the-cob to LOX activity in the germ and cob. Compared to that in legumes like peas and beans, LOX in immature maize seeds was less active by a factor of about 10-fold (Rhee and Watts, 1966).

**DISTRIBUTION IN THE SEED**

Maize LOX was localized mainly in the seed germ, rather than the endosperm (Gardner, 1970). Germination in the dark increased LOX activity considerably, but in the light the increase was somewhat suppressed (Vick and Zimmerman, 1982).
PROPERTIES OF CRUDE LOX ENZYMES

Although the positional specificity of substrate oxidation by maize LOX is relatively well known, very little is known about the properties of the crude LOXs extracted from either quiescent seeds or seedlings. Fresh homogenates of maize seedlings gave a pH optimum of 5.0, but after freezing overnight, the pH optimum was 5.8 (Fritz and Beevers, 1955). This behavior indicated the possible existence of an unstable isoenzyme in the seedlings. The present author has observed that most of the activity is below neutrality and virtually none is above pH 8 in quiescent maize germ. According to B. Axelrod (personal communication), a pH 9.0 activity was demonstrated in germ extracts from quiescent seed, provided that the precaution was taken to use an assay temperature of 10-15°C (the enzyme inactivates at higher temperature), and to add 0.3 mM CaCl₂ and low concentrations (0.625 mM) of linoleic acid plus Tween 20.

A few additional studies of the properties of the crude enzyme were reported. In the presence of substrate, maize LOXs cooxidized chlorophyll (Imamura and Shimizu, 1974). The heat inactivation of LOXs of both immature maize seeds (Wagenknecht, 1959) and germ from mature seeds (Gardner and Inglett, 1971) has been investigated. The production of off-flavors as a result of maize LOX action has been documented by Kalbrenner et al (1974) and Lee (1981).

Leu (1974) identified the main volatiles originating from maize LOX oxidation of linoleic acid as pentanal, hexanal, trans-2-heptenal, ethanal, and heptanal. Compared to soy LOX, maize LOX activity resulted in less 1-pentene, butanal, and pentylfuran and in more ethanal and heptanal.

OXIDATION SPECIFICITY OF CRUDE LOX ENZYMES

The first observation of a 9-specific oxidation by LOX was achieved with a fraction precipitated by (NH₄)₂SO₄ from the germ of quiescent maize seed (Gardner and Weisleder, 1970). As a result, the oxidation specificity of the maize LOX received a disproportionate amount of attention. Oxidation of linoleic acid by the maize germ LOXs incubated at pH values of 6.5-7.4 resulted in 82-89% 9S-LOOH (Gardner and Weisleder, 1970; Hamberg, 1971; Gardner, 1975b). Using a partially purified maize germ LOX, Veldink et al (1972) confirmed the preference for oxidation of carbon-9 of linoleic acid at pH 6.6, but at pH 9, the product was 83% 13S-LOOH. The small amount of 13-LOOH (11-14%) obtained in the oxidation of linoleic acid by maize germ LOX at pH 6.5 and 7.4 was mainly racemic (13R-LOOH), implying that this hydroperoxide arose from autoxidation at these pH values (Hamberg, 1971). By contrast, van Os et al (1979a) found that the 14% 13-LOOH produced by maize LOX at pH 6.6 was mainly 13S-LOOH. Thus, an enzymatic origin for 13-LOOH appeared possible. The control of the ratio of 9S-LOOH to 13S-LOOH by manipulation of the pH indicated the presence of a second LOX isoenzyme or a pH-dependent head-to-tail orientation of the substrate at the active site. The latter possibility is discussed in more detail below.

Egmond et al (1972) completed a comparative study on soybean LOX-I and maize germ LOX regarding the stereochemistry of O₂ insertion. Whereas soybean LOX-I removed the pro-S hydrogen from the carbon-11 methylene of linoleic acid and inserted O₂ on the opposite side of the fatty acid chain to give the 13S-LOOH, the maize germ LOX selected the opposite orientation for H-removal (pro-R) and O₂ insertion (giving 9S-LOOH). To summarize, H-
removal and O₂ insertion were on opposite sides of the molecule for both LOXs. Moreover, these two oxygenations and H-removals are spatially identical if the fatty acid chains are aligned head to tail. Thus, it is plausible that a head-to-tail orientation occurs at the active site of LOX, as postulated in Figure 4. This mechanism may explain why pH dependency on product formation is observed for most LOXs, that is, different affinities of the enzyme for the carboxylate anion versus the carboxylic acid. It could also explain why certain LOXs (e.g., soybean LOX-I) recognize the ω-end, and others (like maize LOX) the carboxylic acid portion of the fatty acid.

The divergence in substrate specificity between maize LOX and soybean LOX-I further defined a dependence on the carboxylic acid group for the former and the methyl end for the latter. Although soybean LOX oxidized linoleic, linolenic, cis-6,cis-9,cis-12-octadecatrienoic acid, and arachidonic acid about equally well, maize germ LOX at pH 7 oxidized linoleic acid best, linolenic acid at 40% of the rate of linoleic acid, cis-6,cis-9,cis-12-octadecatrienoic at <1%, and arachidonic acid not at all (Hamberg, 1971). This data implies that a double bond between the carboxylic acid function and the Δ9 double bond greatly

![Figure 4. Proposed oxidation mechanism of lipoxygenase, showing that oxidation at C-9 (top) and C-13 (bottom) as well as hydrogen removal from C-11 are spatially identical when the fatty acid chains are arranged head to tail at the active site. The model at the top shows initial attack of O₂ from behind the linoleic acid chain and electron transfer via the iron-active site; the model on the bottom shows the completed oxidation product, the hydroperoxide (anion).](image-url)
inhibited oxidation by maize LOX. Also, maize LOX did not oxidize methyl
linoleate.1

As shown in Table II, the oxidation specificity of maize germ LOX at pH 9 was
shifted to the carboxylic acid end of the fatty acid (Veldink, 1971) when
compared to soybean LOX at the same pH (Hamberg and Samuelsson, 1967).
Of course, whether this specificity was due to the same maize LOX isoenzyme
that is active at neutrality or to a different isoenzyme cannot be determined.

Like barley LOX isoenzymes, germinating maize apparently produced an
additional isoenzyme specific for the oxidation of carbon-13. Vick and
Zimmerman (1982) showed that the selectivity for oxidation at carbon-13
increased from 4% in the quiescent seed to 37% after five days of germination.
The apparent isoenzymes of germinating maize have not been separated.

SEPARATION OF AN ISOENZYME

Veldink et al (1972) partially purified maize germ LOX by fractional
precipitation with (NH4)2SO4 followed by gel filtration through Sephadex
G-100. The amount of purification achieved was not clear. Gardner and
Christianson1 nearly achieved isolation of the maize germ LOX. A 312-fold
purification was obtained by the procedure outlined in Table III. The one peak
of activity eluting from the DEAE Sephadex column (Figure 5) was two-thirds
pure, as assessed by PAGE. Only one band of LOX activity was detected at all
stages of isolation, indicating the preponderance of one stable isoenzyme in
quiescent maize germ (e.g., see Gardner et al, 1973). This LOX isoenzyme had a
broad pH optimum between pH 6 and 7.2, with essentially no activity above
pH 8.25.

G. Rice LOX

Flavor deterioration in rice, especially rice bran, has been attributed to LOX,
but the presence of LOX has been reported in rice bran only recently (Shastry

1 H. W. Gardner and D. D. Christianson, communication at the 11th World Congress, International Society of Fat
Research, Goteborg, Sweden, 1972.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Position of Double Bonds</th>
<th>Source of LOX</th>
<th>Carbon of Oxidation (% oxidized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2</td>
<td>9.12</td>
<td>Maize</td>
<td>16</td>
</tr>
<tr>
<td>18:3</td>
<td>9.12,15</td>
<td>Maize</td>
<td>85</td>
</tr>
<tr>
<td>20:2</td>
<td>11.14</td>
<td>Maize</td>
<td>50</td>
</tr>
<tr>
<td>20:3</td>
<td>8.11,14</td>
<td>Maize</td>
<td>40</td>
</tr>
<tr>
<td>20:4</td>
<td>5.8,11,14</td>
<td>Maize</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soybean</td>
<td>100</td>
</tr>
</tbody>
</table>

* Data from Veldink (1971) for maize LOX and Hamberg and Samuelsson (1967) for soybean LOX.
and Raghavendra Rao, 1975). According to Yamamoto and Fujii (1980) and Ida et al. (1983), the LOX activity was localized mostly, if not wholly, in the germ of the bran milling fraction. All 12 varieties of rice examined by Sekhar and Reddy (1982) had LOX activity with a pH 8.0 optimum, except for an acidic optimum in one variety.

Shastry and Raghavendra Rao (1975) detected three isoenzymes of rice by PAGE. They achieved a 10-fold purification by fractional (NH₄)₂SO₄ precipitation. Although they claimed to have eliminated two of the isoenzymes by this procedure, it seems unlikely that their preparation was composed of a

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity (μmoles/min/mg of protein)</th>
<th>No. of Times Purified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Water extract of defatted germ</td>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>2. pH 4.5 supernatant</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>3. (NH₄)₂SO₄ 40–50% saturation</td>
<td>5.0</td>
<td>6.7</td>
</tr>
<tr>
<td>4. Adsorption on Ca₃(PO₄)₂ gel</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>5. DEAE Sephadex A-50</td>
<td>240</td>
<td>320</td>
</tr>
</tbody>
</table>


*Assayed by O₂ uptake and/or conjugated diene absorption at 232 nm.*

Figure 5. Chromatographic separation of partially purified maize germ lipoxygenase by diethylaminoethyl Sephadex A-50 (see Table III). Solid line represents protein absorption at 280 nm and dashed line denotes lipoxygenase activity. (From H. W. Gardner and D. D. Christianson, Lipoxygenase from Zea mays: Purification and characterization. Communication at the 11th World Congress, International Society of Fat Research, Goteborg, Sweden, 1972)
single isoenzyme. This LOX was characterized as having a pH optimum at 8.5, a $K_m$ for linoleic acid of $3.5 \times 10^{-4}$ M, and good stability at 3-5°C. The enzyme was inhibited by 1mM CuSO$_4$ and tryptophan-specific reagents but was not significantly inhibited by either metal chelators or sulfhydryl reagents.

Several investigators have separated LOX isoenzymes from rice bran or germ. Ida et al (1983) separated three isoenzymes (LOX-1, LOX-2, and LOX-3) by fractional precipitation with (NH$_4$)$_2$SO$_4$ (25-50% of saturation) followed by DEAE-Sephadex chromatography. The most active isoenzymes, LOX-2 and LOX-3, were further purified 42- and 57-fold, respectively, by Sephadex G-150. An isoenzyme that appeared to be identical to the LOX-3 of Ida et al (1983) was purified 484-fold by Yamamoto and Fujii (1980) by fractional precipitation with (NH$_4$)$_2$SO$_4$ (30-70% of saturation), heat precipitation of inactive protein (63°C for 5 min), and DEAE Sephadex chromatography. PAGE indicated contamination by only two trace inactive proteins. LOX-3 apparently was also obtained by John et al (1981), who achieved an 85-fold purification.

Properties of the enzyme designated LOX-3 by Ida et al (1983) compared favorably with those of the isoenzyme isolated by either Yamamoto and Fujii (1980) or John et al (1981). The pH optimum was reported at 7.0 (Ida et al. 1983), 6.5-7.0 (Yamamoto and Fujii, 1980), and 6.8-7.0 (John et al. 1981), and the $K_m$ values for linoleic acid (Yamamoto and Fujii, 1980; John et al. 1981) and $9$-linolenic acid were $1.0 \times 10^{-4}$ M and $1.18 \times 10^{-4}$ M, respectively. The $pI$ of LOX-3 was 4.8 (John et al. 1981). The molecular weight of rice LOX-3 was determined to be 95,000 by Yamamoto and Fujii (1980) and 100,000 by John et al. (1981). According to both Yamamoto and Fujii (1980) and John et al. (1981), the isoenzyme was reasonably stable at ambient temperatures or refrigerator storage but was inactive above 50-60°C. Except for 40°C inhibition by 10mM EDTA, no inhibition of LOX-3 was observed with a number of other metal chelators (Yamamoto and Fujii, 1980). Cu$^{2+}$ and Ca$^{2+}$ were without effect. According to John and Lee (1985), the sulfhydryl reagent, $N$-ethylmaleimide, was without effect, but both EDTA and o-phenanthroline inhibited the enzyme.

The presence of Fe$^{2+}$ in LOX-3 was detected by electron spin resonance.

The most effective substrates for LOX-3 were linoleic and linolenic acids; trilinolein and methyl linolate were poor substrates (Yamamoto and Fujii, 1980). According to Ida et al. (1982) and Yamamoto et al. (1980), the oxidation of linoleic acid by this isoenzyme resulted in 83-97% 9-LOOH.

Rice germ LOX-1 and LOX-2 differed considerably from LOX-3 in properties (Ida et al. 1983). The pH optima of LOX-1 and LOX-2 were 4.5 and 5.5, respectively. Both LOX-1 and LOX-2 afforded roughly equal quantities of $9$-LOOH and $13$-LOOH from oxidation of linoleic acid.

H. Rye LOX

Franke and Frehse (1953) were the first to detect LOX activity in rye, but they did not characterize the enzyme. Heimann and Klaiber (1977a) purified rye LOX by fractional precipitation with (NH$_4$)$_2$SO$_4$ (30-50% of saturation) and gel filtration with a Sephadex G-150 column. Isoelectric focusing of the purified LOX revealed multiple bands clustering into two groups at pH 5.1-5.5 and pH 5.8-6.4: thus, a mixture of isoenzymes was indicated. This isoenzyme mixture had a pH optimum at 7.3-7.5 and a molecular weight of 102,000 by gel filtration.
The positional specificity of linoleic acid oxidation was: a 3:2 ratio of 13-LOOH to 9-LOOH at both pH 6.9 and pH 8.8.

Heimann and Klaiber (1977a) further separated the enzyme mixture by a CM-Sephadex C50 column into three isoenzymes, but they did not characterize them individually.

I. Oat LOX

Early reports of oat LOX were communicated by Franke and Frehse (1953) and Popov and Chelitsev (1960). The latter investigators attributed the development of a bitter taste in oat groats to a sequential reaction of lipase and LOX.

Heimann et al (1973a) separated two LOX activities from oats by fractional precipitation with (NH₄)₂SO₄ (25-50% of saturation) followed by Sephadex G-150 gel filtration. One of these LOXs had a molecular weight greater than 250,000 and coincidentally possessed a strong "lipoperoxidase" activity. Lipoperoxidase is discussed in more detail in Section V. The second LOX activity had a molecular weight, 100,000, more typical of other LOXs. This isoenzyme had a weak lipoperoxidase activity and afforded one band by isoelectric focusing (pI = 5.7). Heimann et al (1973b) subsequently showed that the second LOX isoenzyme oxidized linoleic acid to a 88:12 ratio of 9-LOOH to 13-LOOH at pH 7 (pH optimum was 6.75). Heimann et al (1975) investigated oat LOX purified by the method of Heimann et al (1973a) for its propensity to form volatiles from the oxidation of linoleic acid. According to them, the volatiles were 85% hexanal and 15% trans-2-nonenal. By comparison, the volatile aldehydes from oxidation of linoleic acid with soy LOX were 74% hexanal, 17% pentanal, and 0.2% nonanal.

V. HYDROPEROXIDE-REACTIVE ENZYMES

A. Overview

The plant kingdom contains several prevalent hydroperoxide-reactive enzyme systems, and examples of each are found in cereals (Figure 1). Because hydroperoxide lyase cleaves fatty acid hydroperoxides into a shorter chain aldehyde and an aldehyde-acid, this enzyme is important in the genesis of a number of odors characteristic of plants. Hydroperoxide cyclase converts 13-LnOOH into the cyclopentenone fatty acid, 12-oxo-PDA, and in intact plants this metabolite subsequently is transformed via several enzymatic reactions into a plant hormone, jasmonic acid. Cereals contain two different "hydroperoxide isomerase" enzymes. The first of these resides mainly in cereal germ, and converts fatty acid hydroperoxides into α-ketol and γ-ketol fatty acids. The second hydroperoxide isomerase, localized mainly in the endosperm or flour fractions, converts fatty acid hydroperoxides into epoxyhydroxy fatty acids. Subsequently, the epoxyhydroxy fatty acids solvolysize into intensely bitter trihydroxy fatty acids. To avoid confusion, this latter hydroperoxide isomerase will henceforth be called "flour-isomerase." In whole grain preparations composed of both germ and endosperm, the relative importance of the two isomerases can be assessed by the composition of the product mixture.
According to Graveland (1973a), who examined the end products of hydroperoxide conversion in several whole cereal flours, the competition between hydroperoxide isomerase and flour-isomerase is different for each cereal.


**B. Hydroperoxide Lyase**

As shown in Figure 6, hydroperoxide lyase catalyzes the chain cleavage of fatty acid hydroperoxides into an aldehyde and an aldehyde-acid. Those aldehydes possessing a cis-3 double bond are usually isomerized further into aldehydes with a trans-2 unsaturation. Both types of aldehydes also have the potential of being reduced by alcohol dehydrogenase into the corresponding alcohol. These volatile aldehydes and alcohols have potent odors. Hexanal/hexenal and their corresponding alcohols, which originate from 13-LOOH and 13-LnOOH, respectively, have strong grassy or beany odors. Nonenal/nonadienal and their alcohols (from the 9-LOOH and 9-LnOOH, respectively) have been variously described as having melon, cucumber, or violet odors. One of the aldehyde-acid fragments, 12-oxo-trans-10-dodecenoic acid, reportedly has plant wound-healing properties (Zimmerman and Coudron, 1979).

The literature on hydroperoxide lyase in cereals is rather sparse. In cereal grains or seedlings, the enzyme possibly is absent or is present at low levels. The predominance of hydroperoxide isomerase and hydroperoxide cyclase activities in cereals also may mask the presence of lyase. Vick and Zimmerman (1976) reported no lyase activity in barley and maize, but they did not specify the stage of plant development. However, hydroperoxide lyase may reside in green leaves of cereal plants. As assayed by the production of hexanal, cis-3-hexenal, and trans-2-hexenal, Hatanaka et al (1978) demonstrated low levels of lyase activity in both rice and wheat leaves. They also demonstrated that a portion of the

![Figure 6. Chain cleavage of the hydroperoxides of linoleic and linolenic acids catalyzed by hydroperoxide lyase. (Reprinted, with permission, from Gardner, 1985a)](image-url)
activity could be sedimented with the chloroplast fraction. Sekiya et al (1983), who also measured lyase activity by hexanal/hexenal formation, found that the enzyme was located in maize leaves. As reviewed by Gardner (1985a), hydroperoxide lyase generally has been found to be localized in the chloroplasts of green leaves, but in nonphotosynthetic tissue the enzyme is found in organelles other than plastids.

C. Hydroperoxide Isomerase

GENERAL PROPERTIES AND MECHANISM

Hydroperoxide isomerase was discovered in flaxseed by Zimmerman (1966), who reported the formation of an α-ketol from LOOH. Subsequently, isomerase was found in maize germ, and, in addition to α-ketol, another product, the γ-ketol, was identified (Gardner, 1970; Gardner et al, 1975). Thus, with 13-LOOH as the substrate, the products were an α-ketol, 13-hydroxy-12-oxo-cis-9-octadecenoic acid (12,13-ketol) and a γ-ketol, 9-hydroxy-12-oxo-trans-10-octadecenoic acid (9,12-ketol), and with 9-LOOH as a substrate, products were the α-ketol 9-hydroxy-10-oxo-cis-12-octadecenoic acid (9,10-ketol) and the γ-ketol 13-hydroxy-10-oxo-trans-11-octadecenoic acid (10,13-ketol). As noted in the overall pathway of α-ketol and γ-ketol formation (Figure 7), an intriguing transfer of one $^{18}$O from the $^{18}$O$_2$-labeled hydroperoxide occurs. As shown by Veldink et al (1970b) for flaxseed and Gerritsen et al (1976) for maize germ, one oxygen from 13-hydroperoxide (or 9-hydroperoxide) was transferred to a 12-oxo (or 10-oxo) group of both the α-ketol and γ-ketol, but oxygen was not transferred to the hydroxyl group. The hydroxyl group presumably originated from H$_2$O, and, using the isomerase from maize germ, workers demonstrated

![Figure 7. Hydroperoxide isomerase action on the 13-hydroperoxide (left) and 9-hydroperoxide (right) of linoleic acid, showing the fate of oxygens from $^{18}$O$_2$-labeled hydroperoxides.](image)
that a number of reagents (such as methanol, ethanethiol, oleic acid, and linoleic acid) could be substituted for H₂O to form methoxy, S-ethyl, oleoyl, and linoleoyl derivatives (Christianson and Gardner, 1975; Gardner et al., 1975). Furthermore, substitution of H₂O by maize isomerase inverted the stereochemistry of the 9S-hydroperoxide into a 9R-hydroxyl of the α-ketol (Gardner, 1979). The evidence enumerated above suggested a mechanism for isomerase (Figure 8).

Hydroperoxide isomerase and the product ketols have no known function in plants. An early suggestion that ketols may be responsible for certain redox reactions (Zimmerman and Vick, 1970) has not been verified.

The initial product of maize isomerase action on 13-LOOH was recently reported to be the unstable allene epoxide 12,13-epoxy-9,11-octadecadienoic acid, rather than the ketols, which are actually solvolysis products (Hamberg, 1987). Thus, the epoxyallylic cation intermediate shown in Figure 8 may be the immediate precursor of the allene epoxide, but the hydride transfer as shown is unlikely. This hydrogen at C-12 would undoubtedly be lost to solvent as a hydrogen ion during the formation of the allene epoxide.

Figure 8. Mechanism of hydroperoxide isomerase action on (13S)-13-hydroperoxy-cis-9,trans-11-octadecadienoic acid. Structures are abbreviated to show only C-8 through C-14. (Reprinted, with permission, from Gardner, 1985b)
MAIZE ISOMERASE

Isomerase activity was localized mainly in the germ of the maize seed, rather than in the endosperm (Gardner, 1970). In homogenates of the whole maize seed, hydroperoxide isomerase competed well with other hydroperoxide-degrading activities and the ketols accounted for 74% of the products originating from fatty acid hydroperoxides (Graveland, 1973a). Zimmerman and Vick (1970) also reported hydroperoxide isomerase activity in whole-seed homogenates of maize; however, they assayed loss of conjugated diene rather than ketol formation. This method of assay would not discriminate between hydroperoxide isomerase and flour-isomerase. According to Vick and Zimmerman (1982), the hydroperoxide isomerase activity of developing maize seedlings continued to increase in the root/shoot and endosperm/scutellum parts of the seedling for six to eight days, after which time activity declined. Isomerase activity was greater in dark-grown seedlings than in those grown in the light. The authors found an endogenous level of 115-196 ng of α-ketol per seedling, showing that isomerase was active in the intact maize seedlings. After they wounded the seedlings by slicing with a razor blade, the levels of α-ketol increased four- to sixfold within 10 minutes, then declined somewhat. Whether isomerase products have a role in mitigating damage from wounding is not known.

Like most hydroperoxide isomerases, the one from maize germ is membrane-bound. According to Grossman et al (1983), the efficiency of extracting maize isomerase was greatly increased by using 1% Tween 20. The improved solubilization by detergent was consistent with its localization in the membrane. Isomerase extracted from defatted maize germ in the absence of detergent could be purified eightfold simply by sedimentation of the membrane-bound activity between 8,000 and 78,000 × g (Christianson and Gardner, 1975).

Significant progress with further purification of isomerase presumably has been hampered by the membranous nature of the enzyme. A 2.3-fold purification was achieved by (NH₄)₂SO₄ precipitation (0–40% of saturation) (Gardner, 1970). Grossman et al (1983) purified isomerase 3.2-fold from a Tween 20 solubilized extract by passing it through a Sepharose antisoybean LOX column.

The properties of maize isomerase have been sparsely studied. The enzyme was determined to have a pH optimum at 6.6, and a $K_m$ for LOOH of $7 \times 10^{-3}$ M (Gardner, 1970). The sulfhydryl reagents p-chloromercuribenzoate and CH₃HgI were effective inhibitors (Grossman et al, 1983). Apparently, isomerase is active in maize germ maintained under the low moisture conditions of dry storage. The oil in germ flakes did not peroxidize during storage unless isomerase was first inactivated by heat (Gardner and Inglett, 1971).

WHEAT ISOMERASE

A hydroperoxide-decomposing activity in wheat germ extracts was identified as hydroperoxide isomerase by the appearance of the α-ketol product (Zimmerman and Vick, 1970). Christianson and Gardner (1975) observed a sequential conversion of linoleic acid by LOX and hydroperoxide isomerase in wheat germ extracts, and they isolated the products α-ketol, γ-ketol, and 9-linoleoyl-10-oxo-cis-12-octadecenoic acid. The latter compound originates from substitution by linoleic acid instead of H₂O.
In water suspensions of whole wheat flour, only a trace of α- and γ-ketols were observed from sequential action of LOX and hydroperoxide isomerase on linoleic acid (Graveland, 1973a). On the other hand, the flour-isomerase product, principally THA, was abundant, implying that flour-isomerase was in competition with hydroperoxide isomerase for utilization of substrate. When linolenic acid replaced linoleic acid as a substrate in a flour-water suspension, the hydroperoxide isomerase product (α-ketol) was again comparatively minor (Graveland, 1973b). This α-ketol was characterized as 9-hydroxy-10-oxo-cis-12,cis-15-octadecadienoic acid (9,10-ketodiene) and presumably originated from 9-LnOOH.

Very little is known of the properties of wheat isomerase; however, a wheat germ isomerase fractionated by \((\text{NH}_4)_2\text{SO}_4\) had a pH optimum of 6.1 and was inhibited 50% by \(10^{-5} \text{M} \ p\)-chloromercuribenzoate (Zimmerman and Vick, 1970). As in the case of maize isomerase, an essential sulfhydryl group was indicated.

### BARLEY ISOMERASE

The literature concerning barley hydroperoxide isomerase is comparatively extensive, possibly because the oxidation of fatty acids by malting barley causes flavor and taste problems in beer. The principal compounds leading to off-flavors have been identified as \(\text{trans}-2\)-nonenal and 2-methylfurfural; however, no direct link between these volatiles and hydroperoxide isomerase has been established. Inasmuch as \(\text{trans}-2\)-nonenal can originate from 9-LnOOH by hydroperoxide lyase action, this enzyme is more likely to be responsible. Isomers of THA have been identified as the bitter principle in beer, but these compounds originate from the action of a different enzyme, flour-isomerase, on fatty acid hydroperoxides.

Zimmerman and Vick (1970) first noted isomerase activity in barley. The enzyme was principally localized in the germ (Lulai and Baker, 1976; Yabuuchi and Amaha, 1976; Lulai et al., 1981). The extraction of isomerase was facilitated by detergent, indicating that the enzyme may be membrane bound (Yabuuchi and Amaha, 1976; Lulai et al., 1981). Yabuuchi and Amaha (1976) claimed that isomerase activity decreased during germination, but both Schwarz and Pyler (1984) and Lulai et al. (1981) reported increased activity. According to the latter workers, some activity was lost during steeping of the grain, but the enzyme increase during germination was about threefold through the 13th day. In the seedlings, the activity was mainly found in the acrospire (40%) and embryo (48%). In barley flour-water suspensions, hydroperoxide isomerase was competitive with other processes, including flour-isomerase activity; thus, the α- and γ-ketols comprised 53% of the end products of linoleic acid oxidation (Graveland, 1973a).

A number of workers partially purified the barley isomerase, and one investigator claimed to have isolated the enzyme (Führling, 1975). Yabuuchi and Amaha (1976) partially purified the enzyme 22-fold by \((\text{NH}_4)_2\text{SO}_4\) precipitation (0–40% of saturation), chromatography on a DEAE Sephadex column, and gel filtration by Sephadex G-200. According to Yabuuchi (1978), two peaks of isomerase activity were eluted from the DEAE Sephadex column. Führling (1975) purified barley isomerase 118-fold to homogeneity by \((\text{NH}_4)_2\text{SO}_4\) precipitation (0–50%), and a double separation of the activity with Bio-Gel
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A-0.5 m.

More is known of the properties of barley isomerase than of any other hydroperoxide isomerase. The isomerase isolated by Führling (1975) was determined to have a molecular weight of 550,000, 5 mol of iron per mole of enzyme, a pI of 4.69, and a $K_v$ for LOOH of $5 \times 10^{-4}$ M. The pH optimum was found to be 6.6 (Führling, 1975), 6.2 (Zimmerman and Vick, 1970), and 6.8 (Yabuuchi and Amaha, 1976). Barley isomerase was inhibited by Cu$^{2+}$ (Führling, 1975; Yabuuchi and Amaha, 1976), Hg$^{2+}$ (Yabuuchi and Amaha, 1976), nordihydroguaiaretic acid, cysteine, and oleic acid (Führling, 1975). According to Führling (1975), NaCN inhibited the enzyme, but Yabuuchi and Amaha (1976) found no inhibition with KCN; both laboratories agreed that EDTA, iodoacetate, N-ethylmaleimide, and iodoacetamide were without effect. Isomerase was reported to be moderately heat stable (Schwarz and Pyler, 1984).

The distribution of the four possible ketol isomers produced by various barley preparations has been reported in some detail. According to Führling (1975), the ratio of $\alpha$-ketol to $\gamma$-ketol was always 85:15. This ratio of products is approximately the same as for the maize isomerase studied by Gardner (1970). Since the predominant LOX activity of quiescent barley seed was specific for oxidation of linoleic acid at carbon-9, the chief products of sequential action by LOX and isomerase on linoleic acid were 9,10-ketol and 10,13-ketol, which of the total ketol isomers amounted to 67% (Yabuuchi and Amaha, 1976), 90% (Graveland et al, 1972), 70% (Lulai et al, 1981), and 96% (Führling, 1975). During germination, the LOX specific for oxidation of the 13-carbon of linoleic acid increased, thereby resulting in a shift in the ketol isomers. The ratio of 9,10-ketol to 12,13-ketol shifted from 7:3 in quiescent seed to about 4:6 in five-day seedlings (Lulai et al, 1981). Furthermore, Lulai et al (1981) found that ketols originating from the 13-LOOH predominated in the acrospire and rootlet, rather than in the other parts of the seedling. From a barley flour-water suspension spiked with linoleic acid, Graveland et al (1972) isolated products, 9,10-dihydoxy-cis-12-octadecenoic acid (9,10-diol), 12,13-dihydoxy-cis-9-octadecenoic acid (12,13-diol), indicative of having an origin from $\alpha$-ketols. However, the dihydroxy fatty acids did not form when $\alpha$-keto Is were added directly to the barley flour-water suspension.

The formation of volatile aldehydes has been attributed to hydroperoxide isomerase action in barley (Heimann and Timm, 1977b, 1977c; Yabuuchi, 1978), but in tomato the 12,13-ketol was an ineffective substrate for conversion to aldehydes (Galliard et al, 1977). By contrast, Yabuuchi (1978) claimed that hexanal originated from this $\alpha$-ketol. Although isomerase was very active in quiescent seeds, hexanal was not formed unless the enzyme was extracted from seedlings, indicating that an enzyme other than isomerase may have been responsible. Heimann and Timm (1977b) demonstrated a 5% yield of volatile aldehydes by reacting a 1:1 mixture of 9-LOOH and 13-LOOH with a partially purified barley isomerase. The aldehydes were identified as mainly hexanal with traces of trans-2-heptenal and trans-2-octenal. Using purified hydroperoxide isomers, Heimann and Timm (1977c) showed that hexanal and trans-2-octenal were from 13-LOOH, but 9-LOOH was not converted into aldehydes. Heimann and Timm (1977b, 1977c) did not utilize isolated ketols as substrates of aldehyde formation; thus, it would appear that the action of hydroperoxide lyase cannot be discounted in their enzyme preparation.
RYE AND OAT ISOMERASE

The oxidation of linoleic acid by a rye flour-water suspension resulted in about 47% α- and γ-ketols, indicating a fair rate of isomerase activity somewhat comparable to that of barley flour-water suspensions (Graveland, 1973a). Other products were due to competitive reactions, such as flour-isomerase activity.

Heimann and Klaiber (1977a) purified rye isomerase by (NH₄)₂SO₄ precipitation (30-50% of saturation) and gel filtration by Sephadex G-150. The enzyme eluted in the exclusion volume, indicating a high molecular weight. The $K_m$ for LOOH was $3.5 \times 10^{-5}M$.

Since ketols were not produced from oxidation of linoleic acid by an oat flour-water suspension, isomerase appeared to be absent in the quiescent oat seed (Graveland, 1973a); however, Vick and Zimmerman (1979a) demonstrated a fair rate of isomerase activity in oat seedlings. The α-ketol product of oat seedling isomerase was identified by them.

D. Hydroperoxide Cyclase

GENERAL PROPERTIES AND MECHANISM

Hydroperoxide cyclase is a key enzyme in the LOX pathway from linolenic acid to the plant hormone jasmonic acid (Figure 9). This pathway is the best example of a function for LOX in plants. The plant hormone cucurbitic acid, which differs from jasmonic acid only in the replacement of the ketone group by a hydroxy, also may be derived via this pathway.

![Figure 9. Biosynthesis of jasmonic acid from 12-oxophytodienoic acid. (Adapted, with permission, from Vick and Zimmerman, 1983; reprinted, with permission, from Gardner, 1985b)](insert figure.png)
Lipoxygenase Pathway in Cereals

Hydroperoxide cyclase was discovered in flaxseed extracts by Zimmerman and Feng (1978). The enzyme catalyzed the conversion of 13-LnOOH into 8-[2-(cis-pent-2'-enyl)-3-oxo-cis-cyclopent-4-etyl]octanoic acid (Vick and Zimmerman, 1979b). This cyclic fatty acid was given the trivial name, 12-oxo-PDA. According to the latter workers, 9-LnOOH and the hydroperoxides of linoleic acid were not substrates for cyclase. Using 18O-labeled 13-LnOOH, Vick et al. (1980) demonstrated that one 18O was transferred to an adjacent carbon, the 12-oxo group of 12-oxo-PDA. The oxygen transfer resembled the one observed with hydroperoxide isomerase, leading Vick et al. (1980) to propose an epoxy cation intermediate similar to that proposed in Figure 8 for isomerase. The similarity in mechanism coupled with the observation that hydroperoxide cyclase could not be separated from hydroperoxide isomerase by gel filtration and ion exchange chromatography indicated a possible dual identity for the two (Vick and Zimmerman, 1981); however, confirmation of this hypothesis must await further proof.

CEREAL CYCLASES

In cereals, hydroperoxide cyclase activity has been documented by measurement of 12-oxo-PDA in seedlings of barley, maize, oat, and wheat (Vick and Zimmerman, 1979a). Cyclase activity in maize seedlings peaked between four and eight days depending on the conditions of growth (dark versus light) and on the tissue from which it was extracted (the scutellum/endosperm versus the root/shoot) (Vick and Zimmerman, 1982).

Presumably, the presence of 12-oxo-PDA in cereal seedlings may have a bearing on its role as a precursor of jasmonic acid. Vick and Zimmerman (1984) showed that maize, wheat, and oat seedlings metabolize 12-oxo-PDA into jasmonic acid via the pathway shown in Figure 9. Jasmonic acid is probably a previously unrecognized hormone of general importance to plants. The role of jasmonic acid in promoting plant senescence or growth inhibition has been documented (e.g., Ueda and Kato, 1980; Dathe et al., 1981).

It is interesting that most cereal seeds are low in linolenic acid and additionally that in seed the LOX specific for 9-oxidation usually prevails. This set of circumstances would appear to inhibit both the formation of 12-oxo-PDA and jasmonic acid. The process of germination generally causes an increase in the activity of the LOX specific for 13-oxidation, and the shoot portion of the seedling would increase in linolenic acid as a result of chloroplast development. This predicts conditions favorable for increased activity of the jasmonic acid pathway. In fact, the endogenous levels of 12-oxo-PDA increased as a function of germination as well as of exposure of the seedlings to light (Vick and Zimmerman, 1982).

E. Hydroxy Fatty Acids and Lipoperoxidase

An activity, mainly in cereal flours, leads to the formation of hydroxyoctadecadienoic acid (LOH) and hydroxyoctadecatrienoic acid (LnOH) from LOOH and LnOOH, respectively. When catalyzed either by extracts or by water suspensions of wheat flour (Graveland, 1970a), oat flour (Heimann et al., 1973b), or barley flour (Graveland, et al., 1972), the ratio of 13-hydroxy-cis-9,trans-11-octadecadienoic acid (13-LOH) to 9-hydroxy-trans-
cis-12-octadecadienoic acid (9-LOH) was reflective of the ratio of the substrates, 13-LOOH to 9-LOOH. Inasmuch as the formation of LOH from LOOH is competitive with other reactions that utilize LOOH as a substrate, it is not surprising that water suspensions of flours from different cereals afforded a variety of percentages of LOH relative to other products (Graveland, 1973a). The flours of oat and wheat gave the largest yields of LOH, 63% and 33%, respectively; whereas, rye, maize, and barley flours afforded the least, 24%, 19%, and 16%, respectively.

Reduction of LOOH by sulphydryl groups is one of a number of mechanisms proposed to explain the formation of LOH by flour suspensions. Graveland (1970a) suggested that sulphydryl groups in wheat flour-water suspensions could "reduce" LOOH to LOH. However, Mann and Morrison (1975) showed that both added cysteine and N-ethylmaleimide had no effect on the conversion of LOOH into LOH in wheat flour doughs. In the absence of enzymes and other catalysts, cysteine had no effect on LOOH at acidic pHs, but at pHs above 7, LOOH readily was converted into LOH by cysteine (Gardner and Jursinic, 1981). They attributed this reaction to nucleophilic attack by thiolate anion on the terminal hydroperoxide oxygen to give LOH as a leaving group.

On the other hand, cereal "lipoperoxidase" has received the most attention as the probable factor responsible for production of LOH from LOOH. Heimann and Schreier (1970, 1971) reported that an extract from an acetone powder of oat flour converted LOOH into mainly LOH, and they surmised that indigenous phenolic compounds were acting as H-donors for a lipoperoxidase. After the crude oat extract was partially purified by fractional precipitation with (NH₄)₂SO₄ (25-50% saturation), added p-phenylenediamine or pyrogallol served as H-donors in the lipoperoxidase reaction (Schreier and Heimann, 1971). The oat lipoperoxidase had a pH optimum at 6.8 and was inhibited by both CN⁻ and sulfide. Kinetic analyses of the lipoperoxidase purified by (NH₄)₂SO₄ revealed that the enzyme participated in a bimolecular reaction of two one-electron steps with the H-donor, p-phenylenediamine (Heimann et al., 1972). Subsequently, this lipoperoxidase was separated by gel filtration with a Sephadex G-150 column (Heimann et al., 1973a). The two peaks of lipoperoxidase activity, eluting at apparent molecular weights of 100,000 and 250,000, exactly coincided with LOX activity. Compared to the 100,000 protein, the 250,000 protein had much more lipoperoxidase than LOX activity. Further work by Heimann and Klaiber (1977c) showed that lipoperoxidase activity also coeluted with flour-isomerase activity by chromatography with Sepharose 2B, CM Sephadex C-50, and DEAE Sephadex. Isoelectric focusing also failed to separate the two activities. As will become evident in the discussion of flour-isomerase below, this apparent association of LOX, lipoperoxidase, and flour-isomerase may be explained by secondary reactions catalyzed by LOX. For example, Streckert and Stan (1975) found that soybean LOX in the presence of guaiacol converted LOOH into LOH as well as the products normally associated with flour-isomerase, namely EHA and THA. Heimann et al. (1973a) also could not separate soybean LOX activity from soybean lipoperoxidase activity by gel filtration with Sephadex G-150. It is the opinion of this author that secondary reactions of LOX are strongly implicated as the origin of both the lipoperoxidase and flour-isomerase activities. Since one cannot be sure that coelution of activities is indeed due to identical proteins, this interpretation may
prove to be incorrect. Further research is necessary to prove this assertion.

Grosch et al (1971) measured a weak lipoperoxidase activity in wheat, using guaiacol as an H-donor. Since they used the disappearance of conjugated diene as an assay, the formation of LOH would not have been detected.

**F. Flour-Isomerase**

**OVERVIEW**

Flour-isomerase is an enzyme that appears to be restricted to the flour or endosperm portion of cereals, and this enzyme transforms LOOH into EHA and THA. A kinetic analysis of the reaction in wheat flour dough showed that EHA was solvolyzed into THA (Graveland, 1970a). Because solvolysis was rapid in flour-water suspensions, EHA was not observed as an intermediate under those conditions, and thus, most workers have studied only the THA isomers. This area of research has been reviewed by Gardner (1975a, 1980) and Veldink et al (1977).

**PURIFICATION AND PROPERTIES**

The substrates for flour-isomerase were fatty acids (presumably via fatty acid hydroperoxides) such as linoleic acid (Graveland, 1970a) and linolenic acid (Graveland, 1973b). Monoglycerides, which are oxidized by wheat LOX, were not substrates for flour-isomerase (Graveland, 1970a). The conversion of linoleic acid into EHA and THA by wheat flour doughs was confirmed by Mann and Morrison (1975).

According to Graveland (1970a), the transformation of LOOH into EHA and THA (by solvolysis of EHA) was catalyzed by a water-insoluble factor in gluten. By contrast, a water extract of flour did not afford either EHA or THA from LOOH, but LOH was formed instead. Subsequently, Graveland (1970b) showed that the water-insoluble factor was LOX that had adsorbed to the glutenin component of gluten. Thus, the ratio between adsorbed and nonadsorbed LOX correlated with the ratio of EHA-THA to LOOH-LOH. As pointed out by Veldink et al (1977), Graveland found that preformed LOOH was converted to EHA and THA only when the system was anaerobic and linoleic acid was present. This set of conditions strongly implicated the anaerobic reaction of LOX, in which linoleic acid is required to cycle electrons (Figure 3). However, Graveland (1970a) interpreted his results to mean that a reactive intermediate of linoleic acid is specifically required to form EHA and THA.

Graveland (1973a) studied the oxidation of linoleic acid by flour-water suspensions from other cereals and found that flour-isomerase was competitive with other hydroperoxide-reactive enzymes. Since flour-water suspensions were used, the EHA products were completely solvolyzed into THA. Wheat and oat flours yielded the largest percentages of THA, 60 and 25%, respectively, whereas, barley, rye, and maize flours afforded the least at 21, 16, and 2%, respectively.

Oats is the only cereal from which flour-isomerase has been purified and characterized. One of the most interesting aspects of oat flour-isomerase is the inability of investigators to separate flour-isomerase and lipoperoxidase activities. The two activities coeluted from a Sephadex G-150 column (Heimann and Dresen, 1973), and the most active fraction from the Sephadex G-150...
separation was further purified by Heimann and Klaiber (1977c). Lipoperoxidase and flour-isomerase activities continued to coelute after chromatography with Sepharose 2B, Sepharose 6B, CM-Sephadex C-50, and DEAE Sephadex A50. Isoelectric focusing also failed to separate the two activities. By gel filtration on Sepharose 6B, Heimann and Klaiber (1977c) determined that flour-isomerase/lipoperoxidase had a molecular weight of 3,000,000. The high molecular weight of the enzyme and the duality of activities led them to propose an enzyme complex. It is possible that this enzyme complex also contained LOX activity. Earlier research by the same group (Heimann et al., 1973a) showed that the active Sephadex G-150 peak, used as starting material by Heimann and Klaiber (1977c), also had LOX activity. Unfortunately, LOX activity was not reported in their subsequent separations.

A number of other properties of flour-isomerase were determined, such as the effect of inhibitors. LOOH concentrations of $>9 \times 10^{-3} M$ and LOH concentrations of $>3 \times 10^{-3} M$ were inhibitory to flour-isomerase (Heimann and Klaiber, 1977b). Like lipoperoxidase (Schreier and Heimann, 1971), flour-isomerase could be inhibited with $CN^-$ and $Na,S$, and an inhibition by $p$-phenylenediamine also probably was related to its function as an $H$-donor to the lipoperoxidase associated with flour-isomerase (Heimann and Dresen, 1973). Additionally, these workers demonstrated the enzymatic nature of flour-isomerase by heat inactivation at 90°C for six minutes.

MECHANISM OF PRODUCT FORMATION

The data on product formation lead to the conclusion that the mechanism of flour-isomerase is not completely understood. The evidence indicates two possible pathways of product formation by flour-isomerase. As shown by Figure 10, pathway A follows an intramolecular rearrangement mechanism. Thus, rearrangement of 13-LOOH results in mainly trans-12,13-epoxides with minor amounts of cis-12,13-epoxides. Intramolecular rearrangement can occur by either a heterolytic mechanism (Gardner et al., 1984b) or a homolytic reaction (Gardner and Kleiman, 1981). The homolytic pathway leads to an alkoxy radical, which rearranges to an epoxy allylic (Figure 10). The epoxy allylic radical then may combine with molecular $O_2$ to produce epoxyhydroperoxy-octadecenoic acids (Gardner and Kleiman, 1981) or recombine with the hydroxyl radical produced by homolysis of the hydroperoxide as shown in Figure 10. The latter possibility was chosen because it is a reaction known to be catalyzed by soybean LOX (Garssen et al. 1976). Also, cereal LOXs were strongly implicated in the flour-isomerase reaction. However, $O_2$ was consumed during the conversion of LOOH into secondary products by soy LOX and guaiacol (Streckert and Stan., 1975), indicating that combination with $O_2$ may occur to some extent. In certain instances, the epoxy allylic radical may combine with both hydroxyl radical and molecular $O_2$, such as was observed with the conversion of 13-LOOH into EHA by hematin (Dix and Marnett, 1983).

The formation of epoxides by peroxy radical addition to double bonds (pathway B, Figure 10) is a general reaction known for many years. Hydroperoxides can also epoxidize double bonds by a heterolytic mechanism in the presence of certain transition metal ions, such as vanadium (Mercier and Agoh, 1974). In both cases, the result is identical: formation of an epoxide that retains the cis or trans geometry of the parent olefin.
The study of flour-isomerase is additionally complicated by the observation that a specific isomer of EHA does not always solvolyze cleanly by SN2 displacement into one specific isomer of THA. Gardner et al (1984b) and Claeyts et al (1985) showed that a 12,13-epoxy-9-hydroxy-trans-10-octadecenoic acid (12,13-E-9-HA) intermediate was solvolyzed into a 67-78% yield of 9,12,13-trihydroxy-trans-10-octadecenoic acid (9,12,13-THA), with the remainder being recovered as 9,10,13-trihydroxy-trans-11-octadecenoic acid (9,10,13-THA). This indicated that solvolysis occurred partly by an SN1 mechanism, as shown in Figure 11. Furthermore, the positional isomers 9,12,13-THA and 9,10,13-THA could not be separated by chromatography on silica, including silica impregnated with boric acid. However, diastereoisomers of THA could be separated by silica chromatography (Gardner et al. 1984b). Identification of positional isomers of THA by mass spectrometry of the trimethylsilyloxy-methyl ester derivative is also not very reliable, since 9,10,13-THA and 9,12,13-THA give rise to many identical fragment ions. Since the analysis of THA positional isomers has been used to determine the choice between pathways A and B, it is important to be aware of the mechanism of THA formation and the problems encountered with methods of analysis.

Research with flour-isomerase from wheat leads one to conclude that either pathway A or B could be involved. Because wheat doughs produced an 85:15 ratio of both 13-LOOH to 9-LOOH and 12,13-E-9-HA to 9,10-epoxy-13-hydroxy-trans-11-octadecenoic acid (9,10-E-13-HA), pathway A was indicated (Graveland, 1970a). In addition, Graveland (1970a) found an 85:15 ratio of 9,12,13-THA to 9,10,13-THA from analysis of products from KIO₃ oxidation of linoleic acid.

Figure 10. Two alternative pathways proposed for flour-isomerase reaction on the 13-hydroperoxide of linoleic acid. R = CH₁(CH₃)₉ and R' = -(CH₂)₁₁COOH.
the mixture of THA isomers. Markwalder et al. (1975) also found that the oxidation of linoleic acid by wheat flour-water suspensions resulted mainly in 9,12,13-THA. If one presumes that the EHA isomers found by Graveland (1970a) solvolyzed by \( S_N 2 \) substitution, instead of by the mechanism given by Figure 11, then pathway A was again indicated. A few years later, Graveland et al. (1972) claimed that wheat LOX actually oxidized linoleic acid to mainly 9-LOOH instead of 13-LOOH, throwing pathway A into doubt. Subsequently, Graveland (1973b) reported the formation of 9-LnOH, 9,10-ketodiene, and 9,12,13-trihydroxy-trans-10,cis-15-octadecadienoic acid (9,12,13-THDA) from the oxidation of linolenic acid by wheat flour-water suspensions. Since the presence of 9-LnOH and 9,10-ketodiene implied that a 9-specific LOX was responsible for the isomeric distribution of products, the 9,12,13-THDA product indicated pathway B via a putative 12,13-epoxy-9-hydroxy-trans-10,cis-15-octadecadienoic acid intermediate. The structure of the 9,12,13-THDA isomer was determined by mass spectrometry, which is not completely

![Figure 11. Formation of trihydroxyene fatty acids from the 13-hydroperoxide of linoleic acid through an epoxyhydroxyene intermediate. Structures are abbreviated to show only C-8 through C-14.](image)
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reliable for determining isomers. Nevertheless, Graveland (1973b, 1973c) proposed that pathway B was specifically the mechanism involved. Pathway B was also indicated by the cis geometry of the epoxides found by Graveland (1970a).

The relative merits of pathways A and B also are difficult to evaluate after an assessment of the products from barley flour-isomerase. Since a barley flour-water suspension oxidized linoleic acid to an 8:1 ratio of 9-LOOH to 13-LOOH and an 8:1 ratio of 9,12,13-THA to 9,10,13-THA, pathway B was indicated (Graveland et al., 1972). The ratio of THA isomers was determined by analysis of aldehydes from KIO₃ oxidation, which should be a reliable method of analysis. Esterbauer and Schauenstein (1977a) also reported oxidation of linoleic acid by a buffered barley flour suspension that resulted in products indicative of Pathway B. According to them, a 9:1 ratio of 9-LOOH to 13-LOOH and a 9:1 ratio of 9,12,13-THA to 9,10,13-THA was found. The 9,12,13-THA to 9,10,13-THA ratio was determined by chromatography on silica impregnated with boric acid; however, their method only results in separation of diastereoisomers, not positional isomers (see above). Esterbauer and Schauenstein (1977a) also isolated 9,10,11-trihydroxy-trans-12-octadecenoic acid (9,10,11-THA) from their barley preparation, and they concluded that this fatty acid also originated via pathway B through the proposed intermediate, 10,11-epoxy-9-hydroxy-trans-12-octadecenoic acid, which they did not isolate. However, Lulai et al. (1981) obtained direct evidence for pathway A by the oxidation of linoleic acid by an extract of germinated barley. They isolated and identified an isomeric mixture of 12,13-epoxy-11-hydroxy-cis-9-octadecenoic acid and 9,10-epoxy-11-hydroxy-cis-12-octadecenoic acid, which clearly can originate only by intramolecular rearrangement.

The products identified as originating from the action of oat flour-isomerase were indicative of pathway B. According to Heimann et al. (1973b), oat flour-isomerase converted 9-LOOH, from the oxidation of linoleic acid by oat LOX, into the 9,12,13-THA via an EHA intermediate. They identified the EHA intermediate but did not determine its structure. On the other hand, they did determine the structure of 9,12,13-THA by its KIO₃ oxidation products. Heimann and Dresen (1973) also proposed pathway B, but their evidence was insufficient to select between the two possible pathways. Heimann and Klaiber (1977a) gained further evidence for pathway B by demonstrating an intermolecular reaction between 14C-labeled LOH and unlabeled LOOH upon incubation with a partially purified oat flour-isomerase. The 14C-labeled THA product was identified as a radioactive spot on a thin-layer chromatogram with the expected Rₗ value. Using a 4:1 ratio of LOH to LOOH, they calculated that a 56% intermolecular reaction had occurred.

After assessing the cumulative evidence for the flour-isomerases, it is this author's opinion that more work is necessary to determine the relative merits of pathways A and B. Inasmuch as there was, in most cases, a close association between flour-isomerase and LOX activity, the anaerobic reaction of LOX actually may be identical to flour-isomerase activity. It appears certain that the anaerobic reaction of soy LOX operates via pathway A; thus, enough inconsistency exists in the wheat and barley data to throw some doubt on the mechanism. The data for oat flour-isomerase appear to be consistent for pathway B, but it would be reassuring if these data were confirmed.
TRIHYDROXYOCTADECENOIC ACIDS AND BITTERNESS

The various isomers of both THA and LOH elicit bitter tastes. According to Baur and Grosch (1977), an isomeric mixture of 9,10,13-THA and 9,12,13-THA were bitter at a threshold of only 0.6-0.9 \micro mol/ml. The bitterness threshold of a 9-LOH and 13-LOH mixture was 10-fold higher (Biermann et al., 1980). In addition, the isomeric mixture of 9,10,11-THA and 11,12,13-trihydroxy-9-octadecenoic acid (11,12,13-THA) were found to be bitter (Baur et al., 1977).

The LOH and THA isomers accumulate in aqueous cereal preparations by the reactions described above. The incubation of an oat flour-water mixture resulted in an intensely bitter taste, which was attributed by Biermann et al. (1980) to the formation of LOH isomers (750 \mu g/g of flour) and THA isomers (62 \mu g/g of flour). Similar incubations of wheat flour-water mixtures did not give intensely bitter tastes because LOH and THA isomers accumulated in comparatively lesser quantities.

Bitter fatty acids are also found in beer. THA isomers accumulate from the oxidation of linoleic acid during the malting and mashing of barley (Drost et al., 1974); therefore this process may be a major source of THA in beer. Esterbauer and Schauenstein (1977b) isolated 9,12,13-THA, 9,10,13-THA, and 9,10,11-THA from beer in approximately the same isomeric ratios as found during the oxidation of linoleic acid by a barley flour-water suspension (Esterbauer and Schauenstein, 1977a).

THA isomers may also contribute to the stale flavor occasionally found in beer. It is generally agreed that the staleness factor is trans-2-nonenal. Esterbauer and Schauenstein (1977b) found that trans-2-nonenal did not readily form from THA under ordinary conditions but increased after heating and pH decrease in the presence of THA isomers. Formation of trans-2-nonenal from THA isomers during long-term storage of beer seems plausible.

G. Other Products

Oxidation of linoleic acid by flour-water suspensions prepared from wheat, barley, rye, oats, and maize afforded a mixture of 9,10-diol and 12,13-diol in relatively low yield, 2.6-11% of the total (Graveland, 1973a). In addition, 9,12-dihydroxy-trans-10-octadecenoic acid and 10,13-dihydroxy-trans-11-octadecenoic acid were found in small amounts (1.3 and 0.1%, respectively) in the oxidation mixtures from the oat and wheat flours. According to Graveland (1973a), the dihydroxy fatty acids did not originate from \( \alpha \) - and \( \gamma \)-ketois; therefore, their origin is obscure. In barley, the origin of 9,10-diol was apparently from 9-LOOH via an unknown route (Graveland et al., 1972).

Oat and barley flour-water suspensions also afforded a 1-2% yield of 9,10-dihydroxy-13-oxo-trans-11-octadecenoic acid and 12,13-dihydroxy-9-oxo-trans-10-octadecenoic acid from oxidation of linoleic acid (Graveland, 1973a). The 9,10-dihydroxy-13-oxo-trans-11-octadecenoic acid isomer appeared to form from 9-LOOH (Graveland et al., 1972). A plausible route from 9-LOOH could be through intramolecular rearrangement (pathway A, Figure 10) to yield 9,10-epoxy-13-oxo-trans-11-octadecenoic acid (Gardner and Kleiman, 1981), from which the epoxide could solvolyze. The dihydroxyoxo-octadecenoic acids were not converted into THA isomers (Graveland et al., 1972).

The occurrence of 9-oxooctadecadienoic acid and 13-oxooctadecadienoic
acid isomers in flour-water oxidation of linoleic acid was always less than 1% (Graveland, 1973a).

VI. LOX PATHWAY IN PHYSIOLOGY

The notion that the LOX pathway controls certain physiological events in plants is receiving increased attention. It is beyond the scope of this review to detail such research, especially since other recent reviews in this area are available (Gardner, 1985b; Vick and Zimmerman, 1987). In summary, work has primarily focused on plant senescence, control of enzymatic activity, wound response, and plant defense.

The involvement of the LOX pathway in plant senescence has been investigated in several areas. The senescence hormone, jasmonic acid, was briefly discussed above. A role for LOX in the biosynthesis of abscisic acid has also been suggested, but this assertion remains to be demonstrated in a convincing way. Membrane deterioration and inactivation of protein synthesis are reputed to be affected by LOX, and these events are also linked to the senescence of plants.

Douillard and co-workers (e.g., Douillard, 1980, 1981) contend that LOX in conjunction with thioredoxin controls the Calvin and glycolysis pathways by maintaining a balance in the inactive and active forms of enzymes through their conversion into sulfhydryl or disulfide forms. Also, LOX appears to affect the so-called alternate respiration pathway of plant mitochondria through the oxidation of ubiquinone by lipid peroxy radicals (e.g., Rustin et al, 1983).

The wound-healing property of the hydroperoxide lyase product, 12-oxo-trans-10-dodecenoic acid, was discussed above. Products of the LOX pathway may also be produced as defense substances against disease. For example, the rice plant produced 9-LnOH and 13-hydroxy-cis-9,trans-11,cis-15-octadecatrienoic acid in defense against rice blast disease (Shimura et al, 1983; Kato et al, 1984).

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