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Lipoxygenases and the quality of foods

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The significance of plant lipoxygenases for food quality is reviewed, with particular reference to the enzymes from pea and soybean. Various aspects of the sources of the enzymes, their activities, substrate and product specificities, and co-oxidation potential are discussed in the context of food quality and shelf life. The sequences of lipoxygenases, predicted from DNA sequences, from different plants, are compared and the significance of sequence differences assessed in relation to enzyme specificity and the three-dimensional structure of soybean lipoxygenase-1. A novel scheme is proposed for the mechanism of the lipoxygenase-catalysed dioxygenation of polyunsaturated fatty acids in which two different pathways are suggested for the anaerobic and aerobic oxidations.

INTRODUCTION

Oxidative enzymes are of increasing interest to the food scientist because of their effect on both the colour and flavour of plant foods. Polyphenol oxidases are responsible for enzymic browning, often coupled to the loss of vitamin C, in a wide range of vegetables, fruits and juices. Ascorbic acid oxidase also catalyses the oxidation of ascorbic to dehydroascorbic acid, which can then degrade further to browning products. The effects of peroxidases, although visually less obvious, may be equally damaging to food quality as these enzymes in many instances are thermostable and generate free radicals. Another group of enzymes, the lipoxygenases, catalyse the oxidation of polyunsaturated fatty acids to produce hydroperoxides which can be broken down by other enzymes to form desirable and characteristic aroma compounds. Lipoxygenases may also catalyse the co-oxidation of carotenoids, including β-carotene, resulting in the loss of essential nutrients and the development of off-flavours.

Lipoxygenase (EC 1.13.11.12, linoleate: oxygen oxidoreductase) is an iron-containing dioxygenase which catalyses the oxidation of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene units to produce conjugated unsaturated fatty acid hydroperoxides. The occurrence and mode of action of lipoxygenases have been reviewed recently by Gardner (1991), Whitaker (1991), O'Conner and O'Brien (1991) and, as free-radical-generating enzymes, by Donnelly and Robinson (1995). In complex food systems, it has not yet proved possible to define in precise chemical terms how released free radicals react with the very large number of substances present in food materials, although it is becoming increasingly accepted that free radicals may damage texture, flavour and colour. Some free-radical-generating enzymes may withstand thermal processing and therefore may still be sufficiently active in processed foods to initiate changes in quality during long-term storage. Their effects may also be more profound if other protective enzymes have been denatured.

Lipoxygenases have been found in plants, in animal tissues, including marine products, and, more recently, in mushrooms and other fungi. In plants, the enzyme has been found in various organs and a comprehensive list of plant sources where lipoxygenase has been identified has been compiled by Whitaker (1991). Lipoxygenase activity has been reported to be higher in leaves used for making high quality black tea than in those used for lower quality products. Lipoxygenases are of interest to the food scientist, due both to their role in the genesis of volatile flavour and aroma compounds in plant products and to their ability to form free radicals which can then attack other constituents such as vitamins, colours, phenolics and proteins. Lipoxygenase-produced flavour and aroma compounds are desirable in many foods but the enzyme may also give rise to off-flavours, particularly in soybean products. Fujimaki *et al.* (1965) showed that hexanal, derived from the hydroperoxidation of linoleic acid, is primarily responsible for the green-bean-like flavour of defatted soy flour, owing to its extremely low flavour threshold. Interest in lipoxygenase enzymes present in legumes has arisen from their role in the production of volatile compounds associated with grassy-beany and rancid off-flavours in soybean (Rackis *et al.*, 1979). Lipoxygenase-2 (LOX-2) is believed to be the isoenzyme mainly responsible for the generation of *n*-hexanal in

soybeans (Matoba *et al.*, 1985). Enzyme systems degrading linoleic acid and linolenic acid to hexanal and *cis*-3-hexenal, respectively, plus the latter to *trans*-2-hexenal, have been reported in tea leaf chloroplasts (Hatanaka *et al.*, 1976; Sekiya *et al.*, 1976). Off-flavours in sweetcorn have been attributed to lipoxygenase activity (Wagenknecht, 1959) and Koch *et al.* (1959) have claimed that the enzyme is responsible for off-flavour development in cottonseed oil. Furthermore, addition of purified lipoxygenase to blanched peas produced similar off-flavours (Williams *et al.*, 1986). Although heat treatment can be used to inactivate lipoxygenase activity, the functional properties of other protein constituents may be affected. In unblanched stored vegetables, lipoxygenases might also be responsible for the bleaching of chlorophyll. However, during pasta manufacture, the bleaching action on carotenoids can be beneficial.

Soybean seed lipoxygenase 'type I' (LOX-1) (Chan, 1987; Whitaker, 1991) is the best-characterised enzyme. 'Type I' enzymes have an optimum activity at approximately pH 9, whereas 'type II' enzymes, which include soybean seed LOX-2 and LOX-3, are most active between pH 6·5 and 7. It has been reported that LOX-3 is the most abundant isoenzyme in mature soybeans on a protein basis; LOX-1 is almost as abundant, with LOX-2 least abundant. However, LOX-2 has the highest specific activity, so that, on the basis of enzymic activity, similar amounts are present in soybeans (Hildebrand *et al.*, 1988). All of the soybean lipoxygenase isoenzymes are monomeric, have a molecular weight of approximately 100000 and contain one atom of iron per mole of protein.

MODE OF ACTION

Nature of the substrate

Generally the substrate molecule should contain a *cis*, *cis*-1,4-pentadiene moiety with an activated methylene group between the two double bonds. The position of the pentadiene group in the molecule may vary but, for a particular isoenzyme, only one or two of a wider range of substrates are preferred. For example, the best substrate, e.g. linoleic acid, for soybean LOX-1 has the first double bond at carbon atom 6 counting from the methyl end, ω-6 (Holman *et al.*, 1969; Roza & Francke, 1973). However, other molecules containing none of the pentadiene moiety (such as 12-keto-(9*Z*)-octadecenoic acid), the methyl ester (Kuhn *et al.*, 1991*a*), or the activated methylene group, as in the furan derivative (Boyer *et al.*, 1979; Batna & Spiteller, 1994), have been reported to serve as substrates.

The three soybean isoenzymes possess slightly different substrate specificities. LOX-1 is reported to be more active on linoleic acid at the optimum pH of 9. LOX-2 has been claimed to be more active towards arachidonic acid than linoleic acid and LOX-2 and LOX-3 are somewhat more active on methyl linoleate than on linoleic acid (Bild *et al.*, 1977*a*; Axelrod *et al.*, 1981). The lipoxygenases from peas, like soybean LOX-2 and LOX-3, possess greatest activity at pH 6·5 to 7 towards polyunsaturated free fatty acids, their methyl esters, and triglycerides (Christopher *et al.*, 1972; Chen & Whitaker, 1986). Furthermore, soybean LOX-1 has been reported to react with the water-soluble linoleyl sulphate, whereas LOX-2 and LOX-3 showed only limited activity on this substrate (Bild *et al.*, 1977*a*). Recently, it has been shown that soybean LOX-2, unlike the 'type-I' enzyme, oxygenates the esterified polyunsaturated fatty acid moieties in biomembranes thus pointing towards a different action for the two lipoxygenases in soybean cells (Maccarrone *et al.*, 1994).

Nature of the products

Unsaturated fatty acid hydroperoxides are the primary products of the lipoxygenase-catalysed oxidation of the methylene-interrupted polyunsaturated fatty acids which subsequently may be converted both chemically and enzymically into a number of secondary products. Different lipoxygenases from various plant and animal species oxidise polyunsaturated fatty acids stereospecifically. The insertion of molecular oxygen is chiral and positionally specific (Whitaker, 1991). For the oxidation of linoleic acid by plant lipoxygenases, the hydroperoxide group may be located at carbon-9 and/or carbon-13. The chiral forms can be separated by HPLC (Hawkins *et al.*, 1988; Nikolaev *et al.*, 1990). Soybean LOX-1 gave almost exclusively 13S-OOH when linoleic acid was used as the substrate and incubated at pH 9 at 0°C (Roza & Francke, 1973), whereas at room temperature, small amounts of the 9S-OOH were detected. However, greater amounts of 9S-OOH were formed at lower pH values at room temperature and a linear relationship was found between the amount of 9S-OOH formed and the decrease in pH (Bild *et al.*, 1977*a*; Gardner, 1989*a*). The composition of the products formed with different substrates and enzymes under various experimental conditions has been reviewed extensively elsewhere (Vick & Zimmerman, 1987*a*; Gardner, 1991; Whitaker, 1991).

Besides the hydroperoxides, ketodienoic fatty acids have been detected and identified as another group of major reaction products from the incubation of linoleic acid or arachidonic acid with pure pea LOX-1 (Kuhn *et al.*, 1991*b*) and of linoleic acid with pea LOX-2 and LOX-3 (Wu *et al.*, 1995).

The initial products of lipoxygenase activity may be degraded to a variety of products, including several aldehydes, ketones and alcohols, many of which have low flavour thresholds (MacLeod & Ames, 1988). Through the action of hydroperoxide lyases and isomerases, C-9 and C-6 characteristic aroma compounds may be formed (Fig. 1).

The spectrum of volatile compounds isolated from bananas depends on the stage of ripeness of the fruit. Hultin and Proctor (1961) identified 2-hexenal as the main carbonyl compound in banana volatiles. *trans*-2-Hexenal

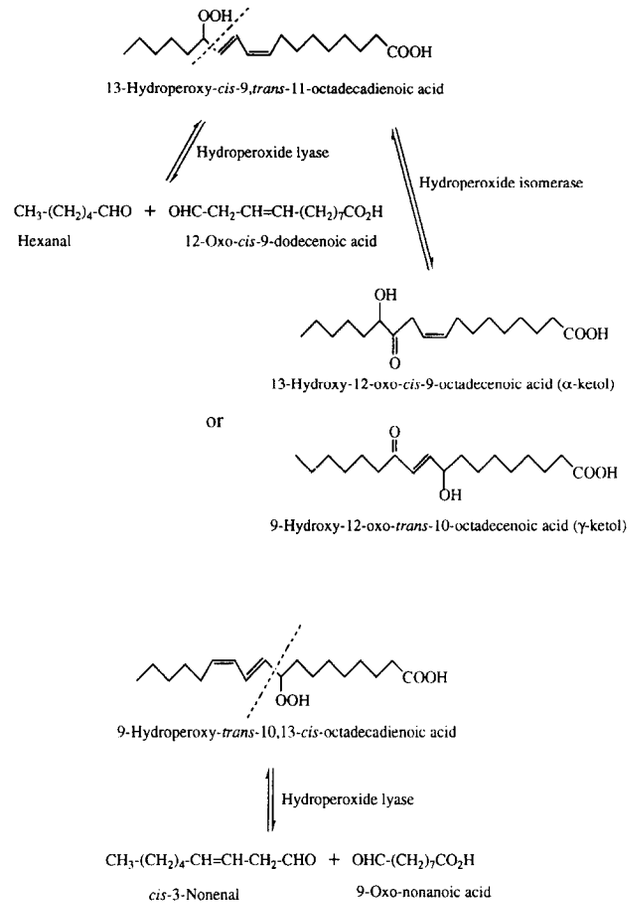

Fig. 1. Possible degradation products arising from the action of hydroperoxide lyases and isomerases.

is also present in banana volatiles (Issenberg & Wick, 1963; Tressl & Jennings, 1972). Stored bananas treated with ethylene have been shown to contain hexanal, *trans*-2-hexenal and 12-oxo-*trans*-dodecenoic acid (Tressl & Drawert, 1973).

Both C-9 and C-6 volatiles are present in cucumber and are believed to be formed from linoleic and linolenic acids (Grosch & Schwarz, 1971). The most important volatile compound in cucumber is believed to be *trans*-2, *cis*-nonadienal (Forss *et al.*, 1962; Fleming *et al.*, 1968). The volatiles are formed during peeling, cutting and chewing, which cause disruption of the tissues. Cucumber peel has been claimed to contain twice as much lipoxygenase activity as the fleshy tissue (Wardale & Lambert, 1980) and a cucumber hydroperoxide lyase has been isolated by Phillips and Galliard (1978). Nona-*cis*,*cis*-3,6-dienal and nona-*cis*-6-enal have been claimed to be important aroma compounds in melons (Kemp *et al.*, 1972, 1974). Three types of enzymes (lipoxygenases, lyases and isomerases) are thought to be involved in their formation (Galliard & Phillips, 1976; Galliard *et al.*, 1976; Vick & Zimmerman, 1987*b*).

It has also been shown that there are other ways by which hydroperoxides can be converted into more stable products. Cleavage of the hydroperoxide O–O bond has been observed with metalloporphyrins (White *et al.*, 1980) and heterolytic cleavage takes place during the reaction of cytochrome P-450 enzymes (Thompson & Wand, 1985). Wilcox and Marnett (1991) have shown, in the presence of porphyrins, that cleavage of the O–O bond can give rise to both heterolytic and homolytic products which include the corresponding alcohols, aldehydes and ketones. More recently another type of enzyme, a peroxygenase, has been described which uses unsaturated acyl hydroperoxides as oxidant and thus catalyses hydroperoxide reduction by a heterolytic mechanism leading to a ferryl-oxo complex analogous to peroxidase compound I. Soybean peroxygenase is a ferrihaemoprotein and has been shown to catalyse both the reduction of 13(*S*)-hydroperoxyoctadeca-9(*Z*), 11(*E*)-dienoic acid (13-HPOD) to 13(*S*)-hydroxy-octadeca-9(*Z*), 11(*E*)-dienoic acid and the formation of 9,10-epoxy-13(*S*)-hydroxyoctadec-11(*E*)-enoic acid (Blee *et al.*, 1993). A cytochrome P-450 enzyme (Song & Brash, 1991) has also been described which dehydrates 13-HPOD to form an allene-oxide as a precursor of ketones and prostaglandin-like compounds. These enzymes, like the hydroperoxide lyases, illustrate a co-operative action with lipoxygenases to form further products from polyunsaturated fatty acids.

Mechanism

A few schemes have been proposed for the mechanism of peroxidation catalysed by lipoxygenases (de Groot *et al.*, 1975; Veldink & Boldingh, 1977; Ludwig *et al.*, 1987; Gardner, 1988) In these schemes, two different pathways involving aerobic and anaerobic reactions have been proposed (Gardner, 1988), although they may occur simultaneously. Three steps are assumed for the formation of the hydroperoxide products: (1) activation of the native enzyme, (2) removal of a proton from the activated methylene group and (3) insertion of the oxygen into the substrate molecule with formation of the hydroperoxide.

It has been demonstrated that the iron in the native enzyme exists predominantly in the high spin Fe(II) form, which does not co-ordinate dioxygen (Feiters *et al.*, 1985; Petersson *et al.*, 1985) and therefore makes it unlikely that this species catalyses hydroperoxidation of the polyunsaturated fatty acids. The Fe(III) form has been claimed to be present in activated lipoxygenases (Cheesbrough & Axelrod, 1983), and is believed to initiate the reaction (Feiters *et al.*, 1985; Whitaker, 1991; Boyington *et al.*, 1993). As the concentration of lipoxygenase-Fe(III) is very small [only about 1% of the total enzyme concentration determined by EPR (Slappendel *et al.*, 1981)], the initial rate of the catalytic reaction is very low. Various lag periods have been observed which depend on the concentration of substrate and enzyme (Funk *et al.*, 1981). The inactive Fe(II) form can be oxidised to the Fe(III) form, and the initial reaction rate increased by added hydroperoxide, such as 13S-OOH for soybean LOX-1. Product analogues lacking the hydroperoxide group, such as hydroxides, or hydroperoxides lacking the long acyl chain or H_2O_2, cannot evoke this rate-enhancing effect (Gibian & Galaway, 1976; Funk *et al.*, 1981; Veldink & Vliegenthart,

1984). Thus the long acyl chain and the hydroperoxide group are crucial for initiating enzymic activity (Gibian & Galaway, 1976). Furthermore, only the predominant hydroperoxide product actually formed by the enzyme can affect the kinetics. The lag phase of soybean LOX-1-catalysed oxidation of linoleic acid appears to be extremely sensitive to 13S-OOH, whereas 9S-OOH is ineffective (Veldink & Vliegenthart, 1984; Vick & Zimmerman, 1987*a*).

The second step has been assumed to be the removal of the hydrogen from the substrate molecule complexed with the enzyme, to form a free radical. The evidence for free radicals is based on anaerobic reduction of Fe^{3+} by fatty acid substrate (Cheesbrough & Axelrod, 1983; Veldink & Vliegenthart, 1984) and the detection of substrate free radicals (de Groot *et al.*, 1973; Chamulitrat & Mason, 1989; Nelson & Cowling, 1990). It is suggested that the loss of the hydrogen occurs before a covalent bond has been established between the substrate and oxygen. The resulting intermediate can be described in two different ways. One scheme requires the elimination of the pro-S ω-8L-hydrogen followed by redistribution of π electrons of the ω-6 and ω-9 double bonds to form a conjugated pentadienyl free radical (Hamberg & Samuelsson, 1967). During the third step the biradical oxygen could then be introduced stereospecifically, when the peroxy radical may finally recapture a hydrogen atom (Hamberg & Samuelsson, 1967). Alternatively, it can be argued that an enzyme-substrate complex may be formed with a Fe^{3+}–carbon bond. Evidence for this is provided by kinetic studies with selected substrates and by development of a chemical model (Corey & Nagata, 1987; Corey & Walker, 1987).

Such a proposed scheme is shown in Fig. 2(a). Previously, it has been suggested that the removal of hydrogen from the methylene group is the same in both aerobic and anaerobic reactions in radical and non-radical pathways. In the new scheme proposed here, the pathway for the anaerobic reaction is the same as reported before, but for the aerobic reaction a transitional three component complex is proposed to be formed between the enzyme, the substrate and the oxygen molecule. Recent structural studies (see later), have shown that the iron in lipoxygenase can complex with six ligands. Four of these ligands are the histidine nitrogen and isoleucine oxygen atoms (Navaratnam *et al.*, 1988; Boyington *et al.*, 1993). The other two ligands might be the double bond in the substrate molecule and the oxygen molecule (Gardner, 1988). The complexing status of the Fe(III) in the enzyme may be expressed as that in Fig. 2(b). Before the removal of the ω-8 hydrogen, it is proposed that there is no interaction between the oxygen and the substrate molecule. However, as the proton is being abstracted by a base moiety of the enzyme, it is proposed that the electron between the hydrogen and the ω-8 carbon is withdrawn to the carbon chain and then moves through the iron bridge in the centre of the complex, to the oxygen. The oxygen, whose reactivity has been increased by the

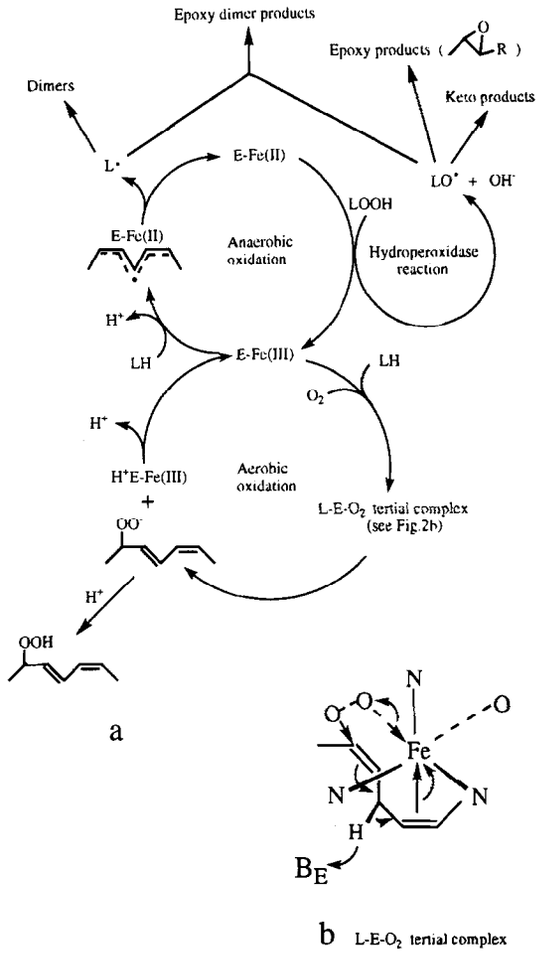

Fig. 2. Proposed scheme for the lipoxygenase-catalysed dioxygenation of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene units under various conditions. (a) Scheme for the whole reaction. (b) Steric structure of the tertiary complex of the fatty acid (abbreviated to show only C-8 through C-14), enzyme and the oxygen molecule. B_E is the base site of the enzyme.

hydrophobicity in the active site of the enzyme (Malmstrom, 1982), will be partially inserted in the ω-6 position before the final removal of the proton. At this stage, a tertiary complex would be formed between the enzyme, substrate and oxygen, in which two partial interactions are present between the base site of the enzyme and the ω-8 hydrogen, and between oxygen and the ω-6 carbon. This is followed by removal of the proton and establishment of a covalent bond between the oxygen and the ω-6 carbon. At the same time the connection between the oxygen and the iron breaks. In this pathway, no radicals are formed. The detected radicals might be the result of the anaerobic reaction and any hydroperoxidase activity of lipoxygenases (Vick & Zimmerman, 1987*a*), or alternatively homolytic cleavage to form lipoxy radicals (Donnelly & Robinson, 1995).

Positional specificity

The positional specificity of the oxygenation is determined by the structure of the enzyme and substrate,

together with the experimental conditions, which can also affect the complexation of the substrate and the lipoxygenase iron. It is often accepted that the position of the activated methylene group from the ω-end may determine the insertion position of the oxygen molecule (Egmond *et al.*, 1972; Gardner, 1989*b*). However, others have assumed that the site of the hydrogen removal, which will then define the position of the insertion of the oxygen, is determined by the distance from the carboxylic group, rather than from the methyl end of the hydrocarbon chain (Kuhn *et al.*, 1985). It has also been claimed that the positional selectivity is, at least in part, due to the conformation of the fatty acid molecule in aqueous media (Chan *et al.*, 1978; Chan & Newby, 1980).

For soybean LOX-1-catalysed oxidation of linoleic acid at pH 9 to 10, the exclusive formation of the 13S-OOH is believed to be due to recognition of the methyl end of the molecule and its access into the cavity of the enzyme (Gardner, 1988). At high pH the substrate molecule is ionised to a carboxylate ion which cannot be accepted by the hydrophobic site in the enzyme. With a decrease in pH more 9S-OOH is formed (Bild *et al.*, 1977*a*; Gardner, 1989*a*), which suggests that the regiospecificity of LOX-1 may be controlled by the carboxylate anion/carboxylic acid ratio of the substrate (Gardner, 1989*a*). Under these conditions, the carboxylic acid group, which is much less polar than the carboxylate group, may be accepted by the hydrophobic site in the enzyme to form an enzyme-carboxylate substrate complex. For each lipoxygenase there are optimum pH values at which the enzyme is most active to produce isomeric products.

Clearly the alternative orientation of the substrate in the cavity of the enzyme has provided a good explanation for the positional effects as well as the stereospecificity of the products. However, it is difficult to find answers from this theory for the positional specificity of the second dioxygenation of arachidonic acid (Bild *et al.*, 1977*b*; van Os *et al.*, 1981), α- and γ-linolenic acids and their methyl esters (Roza & Francke, 1973). According to the cavity and alternative orientation theory, the product specificity will depend on how easy it is for the substrate to enter the cavity in the enzyme; the dominant product will be that in which the hydroperoxy group is at the nearest position from either the methyl end or the carboxylic end, depending on the pH value of the medium. However, the soybean LOX-1 catalyses dioxygenation of α-linolenic acid at pH 9 to yield predominantly a 13-hydroperoxy product rather than the 16-hydroperoxy derivative; likewise the dioxygenation of γ-linolenic acid catalysed by the same enzyme produced the 9-hydroperoxy compound as the main product rather than the 6-OOH derivative (Roza & Franke, 1973). Furthermore, it is well known (van Os *et al.*, 1981) that soybean lipoxygenase catalyses the formation of 15-hydroperoxy-5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid (15-HETE) from arachidonic acid at pH 9. Under certain conditions this 15-HETE can be dioxygenated at a second site; at pH 7·5–8·7, the second hydroperoxy group is located predominantly at carbon 8, with much less at carbon 5 (Bild *et al.*, 1977*b*; Axelrod *et al.*, 1981; van Os *et al.*, 1981). All of these results imply that there are other unknown factors which affect the interactions between the different substrates and enzyme.

Chiral specificity

The chiral-specific oxygenation by soybean LOX-1 produces almost exclusively the S-configuration hydroperoxide, irrespective of the position of the fatty acid carbon atom which is oxygenated (van Os *et al.*, 1981; Kuhn *et al.*, 1991*c*). No reason has been suggested for the exclusive formation of a single isomer. However, it has been reported that an antarafacial relation between the hydrogen being removed and the oxygen being inserted determines the configuration of the hydroperoxide. This has been claimed to be a common feature for this kind of reaction (Hamberg & Hamberg, 1980). The antarafacial relation may be caused by the orientation of the oxygen connected to the iron atom (Fig. 2b). The oxygen position could be affected by basic amino acids attracting the hydrogen atom and be inserted in the substrate molecule antarafacially to the position of the removed hydrogen.

COOXIDATION

Lipoxygenases can be used to bleach carotenoids in wheat flour by a cooxidation reaction which requires the presence of a polyunsaturated fatty acid. The cooxidation activity of lipoxygenases may be source-dependent; lipoxygenases in peas and beans have been reported to have a high cooxidation activity (Grosch *et al.*, 1976) and, for soybean and peas, the 'type II' isoenzymes have been reported to be more effective at cooxidation than 'type I' isoenzymes (Weber *et al.*, 1974). Recently, a high cooxidation activity towards β-carotene and retinyl acetate has been reported for one of two chickpea 'type II' isoenzymes (Sanz *et al.*, 1994). Purified tomato lipoxygenase has been reported to oxidise β-carotene faster than α-carotene and lutein, whereas lycopene, the main tomato pigment, remained unaffected (Cabibel & Nicolas, 1991). Given the choice of natural substrates available and the occurrence of different isoenzymes in any given source, it is not surprising that there have been a number of reports in the literature relating loss of carotenoids as a whole to lipoxygenase activity. Oxidation by lipoxygenases in non-conventional media has been reported by Pourplanche *et al.* (1993).

Lipoxygenases have been claimed to catalyse the oxidation of carotenoids and chlorophyll by a free radical mechanism that requires the presence of a polyunsaturated fatty acid. It is possible that the enzyme is an integral part of the system for cooxidation of carotenoids through the involvement of an enzyme pentadienyl radical-complex (Klein *et al.*, 1984; Ludwig

et al., 1987; Regdel *et al.*, 1985). The cooxidation reaction may arise from abstraction of a hydrogen atom from a carotenoid resulting in the formation of a resonance-stabilised radical which can combine with oxygen to produce carbonyl compounds (Klein *et al.*, 1985). Further products may arise either by decomposition of the radicals or by condensation to form dimers or higher polymers. However, little is known of the chemistry of the degradation products (Cabibel & Nicolas, 1991). One potential mechanism involves the leakage of a peroxyl radical from the enzyme which can then attack the carotenoid, presumably at positions adjacent to double bonds. A second possibility is that an enzyme-bound hydroperoxide is the oxidising species. A third mechanism could be the generation of free radicals in reactions catalysed by anaerobic cycling of lipoxygenase. Whatever the primary mechanism, the net effect generates carotenoid moieties containing a free radical centre which can then react with oxygen to cleave an adjacent double bond to give two carbonyl fragments. However, the oxidised products generally have not been identified yet. In addition to loss of carotenoid colour, such processes, if continued, may lead to the formation of odorous molecules. Some work with tomato lipoxygenase has indicated that cleavage can occur at a number of the double bonds in lycopene and that 6-methyl-5-hepten-2-one is one of the products (Gramshaw, unpublished data). During the apparent associated oxidation of other substances, including thiol groups and inhibitors, it is possible that free radicals are first dissociated from the enzyme. Concentrations of β-carotene higher than 14 μM inhibited oxidation by chickpea lipoxygenases (Sanz *et al.*, 1994), which may be due to the formation of an irreversible enzyme–β-carotene complex as suggested by Cohen *et al.* (1985). Endogenous inhibitors of lipoxygenase in plant sources include chlorophyll, β-tocopherol and phenolic compounds. These substances could act as scavengers for released free radicals and it has been suggested by Regdel *et al.* (1985) that the mechanism for bleaching of chlorophyll differs from that for carotenoids.

STRUCTURE–FUNCTION RELATIONSHIPS

Although lipoxygenases from all sources carry out analogous reactions, the specificity of hydroperoxidation can, as discussed above, vary both in terms of position and chirality; this variation is a function of the nature of the substrate, the reaction conditions and the source of the enzyme. Fourteen complete plant lipoxygenase sequences have been reported, all predicted from DNA sequences. In several cases the overall sequence identities are relatively low, but there is no evidence that many of the sequences predicted from DNA clones correspond to functional enzymes. It has been suggested (Peng *et al.*, 1994) that the sequences fall into two classes, based on their overall amino acid sequence similarities and the nature of the N-terminal sequence; the lipoxygenase from rice leaves shows only ~40% sequence identity to the other 13, is the first reported lipoxygenase sequence containing a leader sequence and is therefore designated the first member of a *lox2* gene class. This adds a further complexity to lipoxygenase nomenclature; as outlined above, the LOX-2 and LOX-3 isoenzymes from soybean seeds are defined as 'type II' sequences and LOX-1 as 'type I'. Under the nomenclature system proposed by the International Commission on Plant Gene Nomenclature (see Shibata *et al.*, 1994), these three soybean isoenzymes fall within the *lox1* gene class. Figure 3 (a–c) shows the alignments of the plant lipoxygenase sequences in the active site regions. It is likely that the control of specificity resides, at least in part, in the nature of the amino acids within the active site. This has been shown clearly in studies of mammalian 12- and 15-lipoxygenases; single amino acid mutations within the 15-lipoxygenase sequence had profound effects on the ratio of 12- and 15-hydroperoxide products (Sloane *et al.*, 1991). The mutations were based on the comparison of human, rabbit, bovine, porcine and rat 5-, 12- and 15-lipoxygenase sequences. A similar comparison of plant lipoxygenases may produce useful information on specificity. All the 14 plant lipoxygenase sequences conserve the essential histidines and the C-terminal isoleucine (Fig. 3), that are ligands to the active site Fe (Boyington *et al.*, 1993), and that are also conserved in mammalian lipoxygenases. In soybean LOX-1, for which a crystal structure has been determined (Boyington *et al.*, 1993), the co-ordinated iron faces two large internal cavities which probably represent the paths for the movement of molecular oxygen and fatty acid substrates to and from the exterior. Sequence comparisons show a high degree of conservation of the 46 amino acids lining the 'fatty acid' cavity. Boyington *et al.* (1993) have described how the structure of the enzyme can affect hydroperoxidation at the 15-carbon of arachidonic acid. Purified potato tuber lipoxygenase catalyses hydroperoxidation at the 5-position of arachidonic acid (Shimizu *et al.*, 1984, 1990); there may be some fundamental difference in the structure of soybean seed 15-lipoxygenase (LOX-1) and potato tuber 5-lipoxygenase that forms the basis of this specificity. The amino acid residue that is involved in the determination of positional specificity in mammalian lipoxygenase (Sloane *et al.*, 1991) corresponds to Phe^{557} in soybean seed lipoxygenase; this residue is valine in the potato, *Arabidopsis* LOX-1 and the rice LOX-2 sequences, a substitution that may be related to the specificity of the enzyme. *In vitro* expression of cloned sequences may provide further information; more work is required to understand positional and chiral specificity, through analysis of the activity and structure of specific mutants produced by recombinant DNA technology. The role of the enzyme in determining position and stereospecificity of hydroperoxidation is important to the directed synthesis of chiral derivatives of hydroperoxides and to understanding the role of hydroperoxides and their metabolites in the determination of food quality.

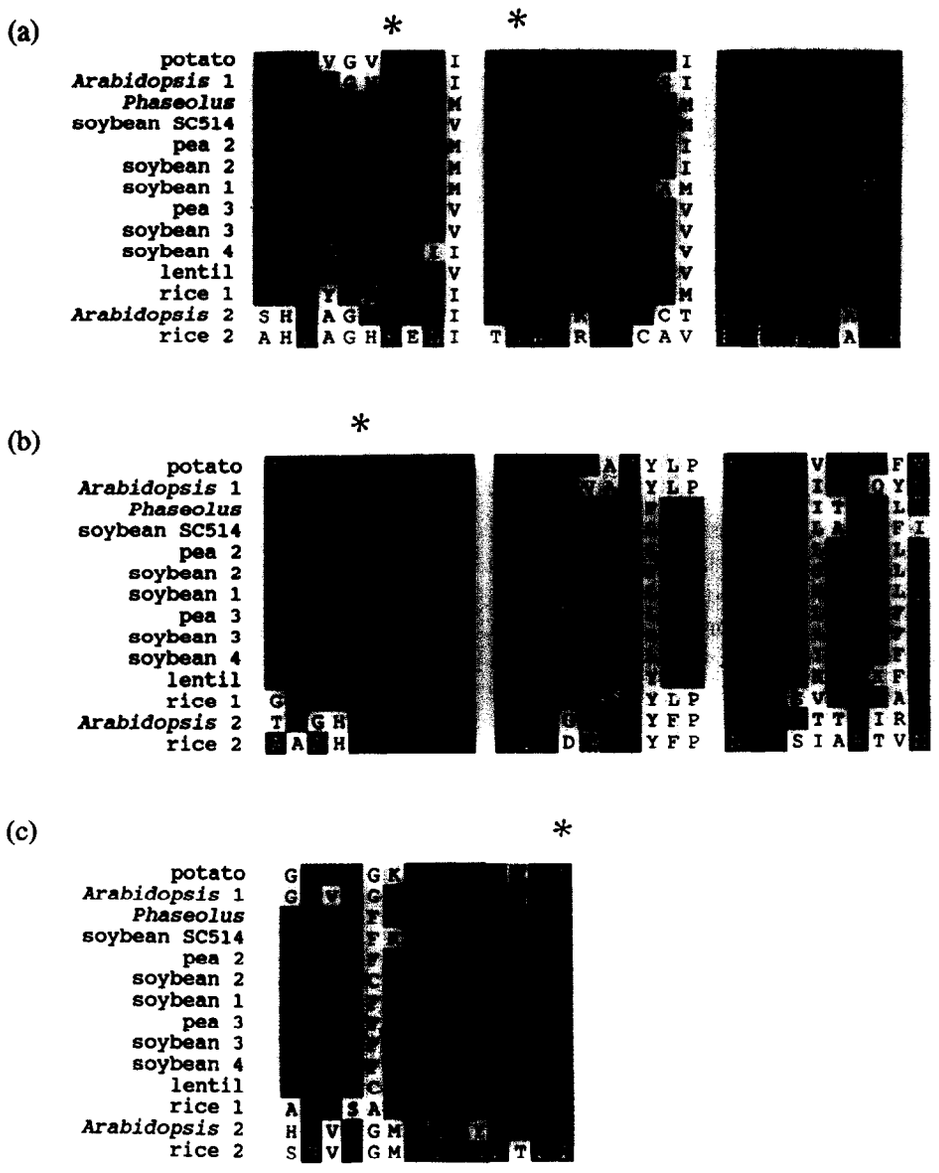

Fig. 3. Comparison of the active site regions of 14 plant lipoxygenase sequences using the University of Wisconsin GCG programmes PILEUP and PRETTYBOX. The programme automatically arranges sequences in order of similarity. The asterisks denote the four residues that contribute iron ligands [(a) His^{499}, His^{504}; (b) His^{690} and (c) the C-terminal Ile^{839}] in the soybean lipoxygenase 1 structure determined by Boyington *et al.* (1993). References: potato, Casey, 1995; *Arabidopsis* 1, Melan *et al.*, 1994; *Phaseolus*, Eiben & Slusarenko, 1994; soybean SC514, Shibata *et al.*, 1991; pea 2, Ealing & Casey, 1989; soybean 2, Shibata *et al.*, 1988; soybean 1, Shibata *et al.*, 1987; pea 3, Ealing & Casey, 1988; soybean 3, Yenofsky *et al.*, 1988; soybean 4, Kato *et al.*, 1993; lentil, Hilbers *et al.*, 1994; rice 1, Ohta *et al.*, 1992; *Arabidopsis* 2, Bell & Mullett, 1993; rice 2, Peng *et al.*, 1994.

MANIPULATION OF FACTORS TO ENHANCE SHELF LIFE

Soybean varieties lacking some or all of the lipoxygenase isoenzymes should theoretically be less susceptible to lipoxygenase-mediated oxidation and the production of undesirable off-flavours. Soybean LOX-1 and LOX-3 are inherited independently of each other (Kitamura *et al.*, 1983) and soybean LOX-2 is closely linked to the LOX-1 gene (Davies & Nielsen, 1986). Naturally-occurring soybean varieties lacking more than one isoenzyme have not been identified (Mack *et al.*, 1987). However, using appropriate crosses, near-isogenic soybean seeds have been developed that lack either isoenzymes-1 and -3 or isoenzymes-2 and -3. Plants grown from seeds lacking two lipoxygenase isoenzymes have shown no obvious deleterious effects when grown under glasshouse conditions (Kitamura, 1984), and the agronomic performance of mutant plants in the field is unaffected by the absence of specific seed lipoxygenase isoforms (Pfeiffer *et al.*, 1992).

In view of the fact that many fruits and vegetables contain not only different types of lipoxygenases, but also a range of lyases and isomerases capable of further degrading hydroperoxides, the manipulation of flavour volatiles should be possible. There are a number of examples in the literature where lipoxygenases have been found to be absent or removed from products.

In some cases, as for soy flour and soy milk, a lack of LOX-2 has resulted in less rancid products (Davies *et al.*, 1987), whereas, for soybean varieties lacking LOX-1, Frankel *et al.* (1988) observed no significant changes.

Homogenates prepared from LOX-1-deficient, LOX-3-deficient and LOX-1 plus -3-deficient soybeans generated significant amounts of *n*-hexanal, but only the LOX-2-deficient line had reduced amounts of hexanal. Removal of LOX-3 increased *n*-hexanal amounts (Hildebrand *et al.*, 1990; Takamura *et al.*, 1991). Furthermore, lines lacking all three isoforms, although not in isogenic backgrounds, still have 70% of the normal amounts of *n*-hexanal (Hajika *et al.*, 1991; Takamura *et al.*, 1991), indicating that there are other pathways to hexanal.

The work of Hildebrand *et al.* (1990) has further indicated that soybean LOX-2 is a more effective enzyme for hexanal formation, although they also claimed that LOX-1 was involved. Furthermore, as larger amounts of hexanal were formed after addition of linoleic acid to soybean extracts, they suggested that lipoxygenases oxidise free fatty acids rather than the naturally occurring esterified fatty acids.

Although lipoxygenases seem to be dispensable in seeds, and a potato variety lacking a specific lipoxygenase isoform has been described (Vaughn & Lulai, 1992), lipoxygenases are likely to be very important in other (non-storage) plant parts. Lipoxygenases are believed to be on the biosynthetic pathways to the plant growth regulators abscisic acid (Parry & Horgan, 1991; Creelman *et al.*, 1992) and methyl jasmonate (Vick & Zimmerman, 1983), both of which play significant roles in plants' responses to environmental stress, including drought, wounding and pest/pathogen attack; in animals, lipoxygenases and other oxidising enzymes such as cyclooxygenases give rise to similar physiologically active compounds.

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