Characterization of authentic recombinant pea-seed lipoxygenases with distinct properties and reaction mechanisms

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The two major isoforms of lipoxygenase (LOX-2 and LOX-3) from pea (*Pisum sativum* L. cv. Birte) seeds have been cloned and expressed from full-length cDNAs as soluble, active, non-fusion proteins in *Escherichia coli*. A comparison of both isoforms purified to apparent homogeneity from *E. coli* and pea seeds has confirmed the authenticity of the recombinant products and established the properties of the native enzymes. Despite 86% similarity at the amino acid sequence level, the enzymes have distinct properties. They have been characterized in terms of specific activity, Fe content, optimum pH, substrate and product

specificity, apparent K_m and V_{max} for the preferred substrate, linoleic acid, and interfacial behaviour with linoleic acid. We have used this evidence, in addition to EPR spectroscopy of the hydroperoxide-activated enzymes and estimates of k_{cat}/K_m , to propose different reaction mechanisms for linoleic acid oxidation for the two isoforms. The differences relate primarily to carbonyl production from linoleic acid for which we propose a mechanism. This implicates the release of a peroxy radical in an aerobic hydroperoxidase reaction, as the source of the carbonyl compounds formed by dismutation of the liberated peroxy radical.

INTRODUCTION

Lipoxygenase (LOX; E.C.1.13.11.12, linoleate: oxygen oxidoreductase) is a non-haem iron-containing dioxygenase that catalyses the oxidation of polyunsaturated fatty acids containing a 1-*cis*, 4-*cis*-pentadiene system, to produce conjugated unsaturated fatty acid hydroperoxides [1,2]. The insertion of molecular oxygen is chiral and positionally specific, and this specificity is dependent on the source of the enzyme. With linoleic acid, LOX from soya bean seeds (LOX-1) is a 13-LOX [2], from potato tubers it is a 9-LOX [3] and from pea seeds [4] it is a 13- or 9-LOX. Analysis of LOX from pea seeds is further complicated because of heterogeneity [5] and the consequent possible contamination of LOX 'purified' from seeds with different isoforms, which copurify but have subtle differences in primary structure. A number of workers [6–10] have studied LOX from pea seeds, but a detailed characterization of the major pea-seed LOX isoforms is required. Production of recombinant LOX offers the potential to evaluate the specificity of the individual isoforms without risk of contamination with other LOX activities.

LOXs are of interest to the food industry because of their effects on food quality [11], and can lead to both desirable and undesirable flavours. They have been shown to have several beneficial effects in breadmaking as a result of both lipid oxidation and co-oxidation reactions [12–14]. The commercial production of hydroperoxides [15], which act as substrates for hydroperoxide lyases, also offers the potential for synthesizing many novel flavour compounds [16]. The availability of single isoforms of recombinant LOX would allow a more refined investigation of the role of LOX in these industrial processes.

Heterologous expression in *Escherichia coli* of developmentally regulated plant LOX has been achieved for soya bean LOX-1 [17], soya bean LOX-3 [18], rice LOX-2 [19], thale cress LOX-1

[20] and lentil LOX-1 [21], and for stress-induced LOX from potato [3] and rice [22]. This should enable their production on a scale for detailed biochemical and spectroscopic analyses, but all except the soya bean LOX are poorly characterized and, in addition, several have been expressed as fusion proteins, or are derived from cDNA clones that are not full-length. Moreover, most native LOX from plant sources are not characterized beyond an examination of their product specificities. There is a need for more thorough biochemical characterization of plant LOX other than soya bean LOX-1, on which most work on LOX structure and mechanism is carried out [23]. This enzyme is easily obtained, but atypical of LOX; it exhibits maximum activity at high pH, has little reactivity with esterified fatty acids and produces secondary compounds only under anaerobic conditions.

The distinction between anaerobic and aerobic reactions of LOX is complicated by the fact that many workers determine LOX activities under conditions which are not strictly anaerobic at the very start of the reaction. The aerobic reaction of LOX has been proposed to occur with [24], and without [11], radical production, but neither accounts for the aerobic production of carbonyl compounds or for aerobic hydroperoxidase activity. Carbonyl production has been described as an anaerobic reaction of soya bean LOX-1 [25] and pea-seed LOX [7]. Most workers, however, studying native LOX from seeds of pea [6,8–10], chickpea [26,27], broad bean [28] and kidney bean [10], from seedlings of lentil [21] and from rabbit reticulocytes [29] have shown that carbonyl production can take place aerobically. The source of the carbonyls is unclear, but they have been explained by the dissociation from LOX of the fatty acid radical following hydroperoxide activation and binding of linoleic acid [10], or the alkoxyl radical immediately following hydroperoxide activation [9]. Others have suggested that preformed hydroperoxides are the source of the carbonyls [6]. The role of some radical mechanism in carbonyl production in the presence or

Abbreviations used: LOX, lipoxygenase; IPTG, isopropyl 1-thio- β -D-galactopyranoside; 13-HPODE, 13-hydroperoxy-(9Z,11E)-octadeca-9,11-dienoic acid; 9-HPODE, 9-hydroperoxy-(10E,12Z)-octadeca-10,12-dienoic acid; 13-HODE, 13-hydroxy-(9Z,11E)-octadeca-9,11-dienoic acid; 9-HODE, 9-hydroxy-(10E,12Z)-octadeca-10,12-dienoic acid.

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absence of oxygen would not appear to be in question, but the apparent differences in the capacity of LOX to retain the radical in the active site would suggest a role for LOX structure in this reaction. LOXs differ considerably in primary structure and requirement for anoxia to produce these compounds, and there would not appear to be a single catalytic mechanism that can account for all LOX behaviour, despite attempts to formulate a single kinetic model [30]; some proposed mechanisms for carbonyl production are hypothetical [21].

An identification of the primary determinants of carbonyl production might be possible through modelling of two LOXs with high similarity at the amino acid sequence level but which differ in this reaction. It may be inappropriate to model soya bean LOX-1 and LOX-3 for carbonyl production because, despite showing 84 % similarity at the amino acid sequence level and having high-resolution crystal structures [31,32], they have different pH optima. A proper understanding of the mechanism of carbonyl production may be gained from comparisons of purified single LOX species and HPLC/GC-MS analysis. We sought therefore to address the mechanism of carbonyl production by full characterization of the two major isoforms of LOX from pea seeds (LOX-2 and LOX-3), which show 86 % similarity at the amino acid sequence level [33,34], after cloning and expression of their full-length cDNAs in *E. coli*.

EXPERIMENTAL

General

DNA sequencing was carried out by Automated Laser Fluorescence, using fluore-dATP (Amersham Pharmacia Biotech; Little Chalfont, Bucks., U.K.), or manually using the Sequenase Version 2.0 DNA sequencing kit. Both strands of all the clones were sequenced. pET3a DNA and glycerol stocks of *E. coli* strains were obtained from Novagen (Madison, WI, U.S.A.). Bacteria were grown in Luria-Bertani medium without glucose and contained, per litre: bactotryptone (10 g), yeast extract (5 g), NaCl (10 g) adjusted to pH 7.0 (NaOH). Agar was added at 1 % (w/v) for plates. Isopropyl 1-thio- β -D-galactopyranoside (IPTG) was from Gibco BRL (Paisley, Scotland, U.K.), carbenicillin (Pyopen) was from East Anglian Pharmaceuticals (Norwich, Norfolk, U.K.), and ampicillin and LOX substrates were from Sigma (Poole, Dorset, U.K.).

Cloning of pea-seed LOX-2 and LOX-3 cDNA for expression in *E. coli*

LOX-2

Full-length LOX-2 cDNA (nt 24–2618 of plasmid pPE320; [34]) was transferred to pET3a [35] in two stages. An *NdeI/EcoRI* and an *EcoRI/BamHI* fragment bearing LOX-2 cDNA nt 24–376 and nt 2594–2618, respectively, were amplified by PCR from pPE320, and both fragments were inserted into *NdeI/BamHI*-digested pET3a. As part of the PCR amplification of the *NdeI/EcoRI* fragment, the codon preference for the first nine amino acids of LOX-2 was optimized [36] by attempting to change the codons for amino acids 3 and 6–9 from CCAAATGTGACAGGACTCCTA to CCGAATGTG-ACCGGCCTGCTG. The resulting plasmid, pETLOX2a, was partially digested with *EcoRI* and ligated with an *EcoRI* restriction fragment from pPE320, corresponding to nt 377–2593 of LOX-2 cDNA. This final construct was referred to as pETLOX2b.

LOX-3

Full-length LOX-3 cDNA (nt 61–2646 of plasmid pPE1036, [33]) was transferred to pET3a [35] in three stages. A *KpnI/BamHI* restriction fragment from pPE1036 bearing nt 61–1912 of LOX-3 cDNA was inserted into M13mp19, in order to introduce an *NdeI* restriction site spanning the ATG start codon of the enzyme, by oligonucleotide-directed mutagenesis [37]. The codon preference for the first nine amino acids of LOX-3 was optimized [36] at this stage by changing the codons for amino acids 3 and 6 from TCAGGCGTGACT to TCTGCGGTGACC. An *NdeI/BamHI* restriction fragment from the resulting plasmid, M13LOX3a, was then inserted into pET3a. The resulting plasmid, pETLOX3a, was digested with *BamHI* and ligated with a *BamHI* fragment bearing nt 1913–2646 of LOX-3 cDNA amplified by PCR from pPE1036. As part of the PCR amplification of this fragment, the termination codon was also changed from TGA to TAA; the former can be mistranslated as tryptophan in *E. coli* [38]. This final construct was referred to as pETLOX3b.

Expression and detection of LOX-2 and LOX-3 immunoreactive polypeptides

Plasmids pETLOX2b and pETLOX3b were transferred into BL21(DE3)pLysE cells [39] for protein expression. Culture medium (Luria-Bertani medium without glucose containing 200 μ g/ml carbenicillin) was inoculated with an overnight culture in ampicillin (50 μ g/ml) and incubated at 37 °C until the cell density had increased to A_{600} 0.6. IPTG (0.4 mM final concentration) was then added and the culture transferred to 20 °C for a further 20 h.

To separate soluble from insoluble proteins, whole cells were centrifuged at 5000 *g* for 5 min. The pelleted cells were homogenized in 50 mM sodium phosphate buffer (pH 6.8), passed through a French Press (6.9 mPa) and centrifuged at 10000 *g* for 10 min; the supernatant was designated the soluble fraction and the pellet, resuspended in phosphate buffer, the insoluble fraction. Samples were diluted with an equal volume of 2 $\times$ SDS/PAGE buffer (0.1 M Tris/HCl buffer, pH 6.8 containing 4 % [w/v] SDS, 20 % [v/v] glycerol, 0.2 % [w/v] Bromophenol Blue and fresh 0.2 M dithiothreitol) and heated at 95 °C for 3 min. For markers, authentic pea-seed LOXs were extracted from finely ground dry seed (*Pisum sativum* L. cv. Birte) in 1 $\times$ SDS/PAGE sample buffer (100 μ l/mg powder) and heated as above. Polypeptides were separated on SDS/PAGE gels (12 % [w/v] resolving; 4 % [w/v] stacking; acrylamide:bisacrylamide, 200:1) using a discontinuous buffer system [40]. For immunodetection, polypeptides were transferred to nitrocellulose [41] and probed with pea-seed anti-LOX IgG (anti-B, [5]). LOX IgG on protein blots was detected using goat anti-rabbit IgG-alkaline phosphatase conjugate and the substrate 5-bromo-4-chloro-3-indolyl phosphate with Nitro Blue Tetrazolium. Marker proteins on blots, stained with 0.2 % (w/v) Ponceau S in 3 % (w/v) trichloroacetic acid/sulphosalicylic acid, were used to determine apparent M_r of unknown proteins.

Purification of pea seed LOX-2 and LOX-3 from *E. coli* and pea seeds

Crude *E. coli* soluble protein fractions containing recombinant LOX-3 were purified by chromatofocusing on a Polybuffer Exchanger 94 column (1.6 cm $\times$ 90 cm) or an FPLC Mono-P column (0.25 cm $\times$ 20 cm) equilibrated with 25 mM Bistris buffer,

pH 6.7. Unbound material was discarded and bound material eluted with a mixture of 4% (v/v) Polybuffer 96/6% (v/v) Polybuffer 74 (pH 5.5); fractions containing LOX activity were pooled and concentrated by ultrafiltration (YM30 membrane, Centrplus-50 and Centricon-50, Amicon; Stonehouse, Gloucestershire, U.K.). This sample was estimated by SDS/PAGE to be about 95% pure. Initial purification of the crude fraction prior to chromatofocusing was occasionally achieved by diafiltration of the sample against 50 mM sodium phosphate buffer (pH 6.8), followed by anion-exchange chromatography on DEAE-Sepharose CL-6B. Further purification to apparent homogeneity was achieved by gel-filtration on Superdex 200 (25 mM histidine/HCl buffer [pH 6.5] containing 0.15 M NaCl, 1 mM dithiothreitol and 1 mM EDTA) followed by anion-exchange chromatography on Mono-Q (0–0.35 M NaCl gradient, 25 mM histidine/HCl buffer [pH 6.5] containing 1 mM dithiothreitol and 1 mM EDTA). Enzyme was desalted on Sephadex G25 into 25 mM Bistris buffer (pH 6.7), concentrated in a Centricon-50 and stored either as small aliquots by flash-freezing in liquid nitrogen or in ammonium sulphate (50% saturation) at 4 °C.

LOX-3 from pea seeds was partially-purified using the method of Wu et al. [4]. Further purification to apparent homogeneity was achieved after anion-exchange on DEAE-Sepharose CL-6B and chromatofocusing on FPLC Mono-P using the elution conditions for the recombinant enzyme. The final product was lyophilized and stored at –20 °C.

LOX-2 from *E. coli* and pea seeds were purified to apparent homogeneity and stored as LOX-3 except for a number of alterations. These included the use of Bistris buffer at pH 6.3 instead of pH 6.7 and the omission of EDTA. In chromatofocusing, LOX-2 did not elute from the column at pH 5.5; when all bound protein had been eluted at this pH, LOX-2 was eluted using the same Polybuffer mixture at pH 4.5. Further purification of LOX-2 to apparent homogeneity was similarly achieved by gel-filtration on Superdex 200 and anion-exchange chromatography on Mono-Q.

Determination of properties of pea-seed LOX-2 and LOX-3 from *E. coli* and pea seeds

Preparation of linoleic acid and determination of specific activity

For the standard procedure, linoleic acid (16.8 μ l, 1 mg/ml) was dispersed by sonication (18 μ , 3 $\times$ 30 s, Soniprep 150; MSE, Leicester, U.K.) in 50 mM sodium phosphate buffer (pH 6.8; 2 ml) containing 1% (v/v) Tween 20 to give a 30 mM stock solution ($A_{600} < 0.05$). This method was optimized for LOX activity and was well characterized (see below) for the reasons outlined in Schilstra et al. [42]. A working dilution for all assays (1 ml) was prepared by diluting 0.5 ml of the stock solution in a total volume of 50 ml of 50 mM sodium phosphate buffer (pH 6.3; $A_{234} 0.2 \pm 0.02$), to give 0.3 mM linoleic acid in 0.01% (v/v) Tween 20. Linoleic acid solutions were prepared fresh daily and not stored. Hydroperoxidation (hydroperoxide and hydroxy acid production) was determined by an increase in A_{234} . One unit of LOX activity is the amount of enzyme required to produce 1 μ mol of hydroperoxide and hydroxy acid per minute at 25 °C (pH 6.3; ϵ of each, 25 mM⁻¹·cm⁻¹; Z. Wu, R. K. Hughes, R. Casey and D. S. Robinson, unpublished work). Initial rates of hydroperoxidation were calculated from the linear portion of the progress curves. Soluble protein was estimated using the Bradford assay [43] with BSA as a standard.

The specific activity of LOX-2 was calculated from at least four dilutions of the enzyme over which there was a first-order relationship between the rate of linoleic acid hydroperoxidation and enzyme concentration in the assay (0–30 μ g of LOX/ml).

For LOX-2, hydroperoxides accounted for virtually all (> 80%) of the products of linoleic acid oxidation (Z. Wu, R. K. Hughes, R. Casey and D. S. Robinson, unpublished work), so the rates of linoleic acid hydroperoxidation, measured at 234 nm, and transformation, defined as conversion to hydroperoxides, carbonyls and hydroxy acids, are assumed to be identical.

The specific activity of LOX-3 was calculated from the amount of protein that produced $A_{234} 0.01/\text{min}$, as the rate of linoleic acid hydroperoxidation was not first order with respect to enzyme concentration in the assay. It was necessary to measure the activity of a range of dilutions of the LOX extracts and extrapolate to zero enzyme to obtain an accurate representation of specific activity. For this isoform, a significant proportion (up to 67% at pH 6.3) of the products of linoleic acid oxidation were equimolar amounts of carbonyls and hydroxy acids, which possess the same molar extinction coefficient ($\epsilon = 25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) at 234 nm as the unsaturated hydroperoxides (Z. Wu, R. K. Hughes, R. Casey and D. S. Robinson, unpublished work). Carbonyl production was dependent on pH and enzyme concentration in the assay. LOX concentration could be used, where applicable, to estimate the relative proportions of carbonyls, hydroperoxides and hydroxy acids; 2 mol of hydroperoxide yielded 1 mol of hydroxy acid and 1 mol of carbonyl. For pure LOX-3, $A_{234} 0.01/\text{min}$ (or $4 \times 10^{-4} \mu\text{mol}$ of hydroperoxide formed/min) corresponded to the activity from approximately 0.01 μ g of LOX. Carbonyl production using this amount of protein in the assay was negligible; as carbonyls were produced together with hydroxy acids, the rates of linoleic acid hydroperoxidation measured at 234 nm and transformation can be assumed identical. A unit of carbonyl-producing activity was the amount of enzyme required to produce 1 μ mol of carbonyl per minute at 25 °C (pH 6.3).

Kinetic parameters

Kinetic parameters of linoleic acid hydroperoxidation for pea LOX-2 and LOX-3 from *E. coli* and pea seeds were determined. Oxygen could be assumed to be saturating for measuring initial reaction rates, which were approximately first order at low substrate concentrations, such that one-, rather than two-, substrate kinetic analyses could be applied to determine kinetic parameters. Only apparent K_m and V_{max} values could be determined since the linoleic acid was not in true solution at neutral pH. The first-order rate constant, k_{cat} , is defined as the number of mol of linoleic acid transformed per s per mol of active sites (assuming all substrates are at their saturating concentrations and 1 active site per LOX molecule of $M_r 97400$).

Substrate specificity

The relative activity of LOX-2 and LOX-3 from *E. coli* and pea seeds was determined with linoleic acid, linolenic acid, arachidonic acid and methyl linoleate. All substrates were prepared as 30 mM stocks and assayed at 0.3 mM (pH 6.3) and 25 °C, following the standard procedure with linoleic acid. Methyl linoleate could not be dispersed effectively by sonication and was prepared as an emulsion in acetone/ethanol/phosphate buffer (1:1.5:97.5, by vol.) [8].

Product specificity

Identification of the products of LOX-2- and LOX-3-catalysed oxidation of linoleic acid was carried out by HPLC/GC-MS [4]. Briefly, reaction mixtures were acidified and extracted with diethyl ether, dried with sodium sulphate and the residue redissolved in hexane for HPLC purification. For GC-MS

analysis, hydroperoxylinoleic or hydroxylinoleic acid peaks were trimethylsilylated [4].

Protein sequencing

The N-terminal sequence of purified pea LOX-3 from *E. coli* and pea seeds was determined. Samples were separated on 12% (w/v) SDS/PAGE gels and transferred by semi-dry blotting to PVDF membrane (Immobilon P, Millipore; Watford, Herts., U.K.) in 10 mM CAPS [3-(cyclohexylamino)propane-1-sulphonic acid] buffer (pH 11.0), containing 10% (v/v) methanol, and rinsed thoroughly in deionized water. The blot was stained with 0.1% (w/v) Coomassie Blue G250 in 50% (v/v) methanol and destained with 50% (v/v) methanol. Bands were excized and N-terminally sequenced by Edman degradation using an Applied Biosystems model 491 Procise protein sequencer (Warrington, Cheshire, U.K.) in the pulsed-liquid mode.

EPR spectroscopy

The catalytic-iron environment of recombinant LOX-2 and LOX-3 was probed by EPR spectroscopy of the resting enzymes and after activation with 13-hydroperoxy-(9Z,11E)-octadeca-9,11-dienoic acid (13-HPODE) or 9-hydroperoxy-(10E,12Z)-octadeca-10,12-dienoic acid (9-HPODE). Hydroperoxides were prepared from the reaction of linoleic acid with soya bean LOX-1 (Sigma) or purified tomato 9-LOX, respectively, purified and identified by HPLC/GC-MS [4], and stored in ethanol at -20°C under nitrogen.

Low-temperature EPR measurements were carried out using a Bruker ER200D-SRC EPR spectrometer equipped with an Oxford Instruments ER900 liquid-helium gas-flow cryostat. All spectra were recorded at 10 K with 5 mW microwave power and 9 gauss (0.9 mT) modulation amplitude; conditions which avoid saturation and modulation broadening. EPR active iron content was estimated using 1.98 mM Cu^{2+} EDTA as standard. Corrections were made for the different spin states and populations [44]. Experimental conditions are given below.

Total iron analysis

Samples of LOX-2 (3×1 mg) and LOX-3 (2×4 mg) used for EPR were digested [45] for determination of total iron content by inductively coupled plasma-emission spectrometry (Thermo Unicam, Cambridge, U.K.). Buffer controls put through the same digestion procedure were used to determine the amount of iron from contamination.

RESULTS AND DISCUSSION

Cloning of LOX-2 and LOX-3 cDNAs

The cDNAs encoding the two major forms of pea-seed LOX (LOX-2 and LOX-3) were cloned into pET3a. Sequencing of the insert in pETLOX3b indicated that the intended changes in codon preference for amino acids 3 and 6, and the termination codon, were present. The predicted amino acid sequence of recombinant LOX-3 was identical to that predicted from pPE1036 (EMBL database accession no. S01142; [33]).

The predicted amino acid sequence of recombinant LOX-2 had three changes from that predicted from the published sequence of pPE320 (EMBL database accession no. S07075; [34]). Two of these (L333 $\rightarrow$ I and M561 deletion) represent errors in the sequence of pPE320 in the database and have been reported before [46]. Sequencing of the insert in pETLOX2b indicated that the intended changes in codon preference for

amino acids 6–9 were present; a mutation (P3 $\rightarrow$ T) was detected in this expression construct and represented the only difference in predicted amino acid composition of LOX-2 from pea seeds and *E. coli*. This was not intended, but apparently did not affect function (see below).

Expression of pea-seed LOX-2 and LOX-3 in *E. coli*

Pea-seed LOX-2 and LOX-3 were expressed as active, soluble non-fusion proteins in *E. coli*. Immunoreactive polypeptides that comigrated exactly on denaturing gels with their counterparts from pea seeds were detected in crude soluble protein extracts. There was no expression from a control strain bearing the expression vector without a LOX insert (Figure 1a). Recombinant LOX-3 migrated as a single polypeptide of M_r approx. 97000, as predicted from the cDNA sequence (M_r 97134; [33]); despite LOX-2 having about the same predicted molecular mass as LOX-3 (M_r 97628; [34]), this polypeptide migrated anomalously as a polypeptide of M_r approx. 94000.

Purification of LOX-2 and LOX-3 from *E. coli* and pea seeds

LOX-2 and LOX-3 from *E. coli* and pea seeds were purified to apparent homogeneity by a combination of anion-exchange chromatography, chromatofocusing and gel-filtration. Purified recombinant LOX-3 was detected in Coomassie-stained SDS/PAGE gels as an apparently single polypeptide (Figure 1b), but occasionally as two closely migrating polypeptides of about the same M_r with smaller proteolysed fragments (results not shown). The corresponding purified sample from pea seeds was always detected as two products (Figure 1b). The relative proportions of the two products from both sources varied. N-terminal sequencing of LOX-3 from both sources indicated that various non-specific modifications (short deletions of up to the first 43 amino acids) had occurred. Heterogeneity of pea-seed LOX has been demonstrated at the N-terminus [5], which is a highly variable region amongst plant LOXs [2]. Modelling of pea LOX-3 against the crystal structure of soya bean LOX-1 [31] indicated that the major cleavage site 43 amino acids from the N-terminus of LOX-3 (producing the faster-migrating product in the doublet) was between the first and second strands of the N-terminal β -barrel, in a large, flexible loop containing many glycine residues, which were probably disordered and prone to proteolysis. Protein-blot analysis of purified LOX-3 from both sources indicated that they were slightly proteolysed and a range of very minor immunoreactive polypeptides (M_r approx. 40000–90000) were detected (results not shown).

LOX-2 appeared as a major polypeptide of M_r approx. 94000 in Coomassie-stained SDS/PAGE gels from both sources (Figure 1b). Minor polypeptides detected on occasion in these gels of LOX-2 samples from both sources (shown for recombinant enzyme only), which co-migrated with those detected on protein blots (results not shown), indicated that these were proteolysed LOX fragments and that the samples were homogeneous. The P3 $\rightarrow$ T amino acid mutation in LOX-2 from *E. coli* was not predicted to affect function. Thus, from a comparison of the crystal structure of soya bean LOX-1 [31], the region of the protein containing this mutation, which is absent from mammalian LOX, is predicted to be non-essential for substrate interactions, and recombinant LOX-2 had indistinguishable properties from its counterpart in pea seeds (see below).

Biochemical properties of recombinant and authentic pea-seed LOXs

Purified LOX-2 from *E. coli* or pea seeds had a specific activity

Figure 1 Protein-blot analysis and purification of LOX-2 and LOX-3 from *E. coli* and pea seeds

(a) Crude soluble protein extracts from pea seeds and *E. coli* BL21(DE3)pLysE, transformed with pET3a (control plasmid), pETLOX2b or pETLOX3b, were separated on SDS/PAGE gels, blotted and probed with anti-B [5]. (b) Coomassie-stained SDS/PAGE gels of LOX-2 and LOX-3 purified to apparent homogeneity from *E. coli* and pea seeds. Dashed arrows indicate position of proteolysed immunoreactive LOX-2 polypeptides.

of 1.2–1.8 μmol of linoleic acid transformed/min per mg of protein. Purified LOX-3 from *E. coli* or pea seeds had, on average, a 29-fold higher specific activity, of approx. 40–48 μmol of linoleic acid transformed/min per mg of protein. For linoleic acid hydroperoxidation, LOX-2 enzymes exhibited first-order behaviour in the presence of detergent; in the absence of detergent, the enzymes saturated the assay and the hydroperoxidation rate was reduced, although less than for LOX-3 (Figure 2a). The rate of hydroperoxide production by LOX-2 enzymes in the absence of detergent was very dependent on enzyme concentration in the assay (Figure 2a); pH was also critical and activity was detected only when the stock solution of linoleic acid was prepared at pH 6.3 and not at pH 6.8.

LOX-3 enzymes always saturated this assay. In the presence of detergent this was very rapid; in its absence, hydroperoxidation rate and the level of saturation for both enzymes was significantly reduced but relatively independent of enzyme concentration in the assay (Figure 2b). These interfacial effects in LOX catalysis are well established [42], and the kinetic differences observed probably relate to the form in which linoleic acid, which is not in true solution at neutral pH, is presented to these enzymes. The effects of micelle size distribution (detergent and fatty acid), levels of free or monomeric fatty acids, their concentrations and buffer pH are likely to be significant [42]. In the present study, the assays were performed well above the critical micelle concentration of linoleic acid (< 20 μM at pH 7.0; [47]), but well below the $\text{p}K_a$ of linoleic acid (7.9; [48]) and the critical micelle concentration for Tween 20 (6% [w/v] at 25 $^{\circ}\text{C}$; [47]). This would suggest that micellar associations of linoleic acid with detergent monomers determined activity with this substrate. Near-identical rates of linoleic acid hydroperoxidation in the absence of detergent monomers for LOX-2, but not LOX-3, enzymes suggested a difference between the two enzymes in how the substrate was presented to the fatty acid pocket. Nevertheless, linoleic acid must have reacted with both isoforms as a monomer

and these were probably in association with detergent monomers or fatty acid micelles by a simple reversible-equilibrium process [42]. Interfacial effects in LOX catalysis make comparison of the specific activities reported in the present work with those determined for other purified LOX, using different substrate-solubilization methods, inappropriate.

LOX-2 from *E. coli* and pea seeds had identical apparent K_m (0.3 mM) and V_{max} (0.01 $\mu\text{mol}/\text{min}$) values for linoleic acid hydroperoxidation (Table 1). These were 22- and 35-fold lower, respectively, than the corresponding values for the LOX-3 enzymes (Table 1) and suggested that the fatty acid pocket of LOX-2 had a significantly higher affinity for linoleic acid than LOX-3. A suitable comparison of K_m linoleic acid for purified LOX in other studies is not possible, as the substrate-solubilization methods used vary widely; in the present study, the apparent values for K_m and V_{max} were dependent on only very subtle changes in the substrate-solubilization method, although they were always different for LOX-2 and LOX-3 by a similar order of magnitude. For both isoforms, first-order relationships at low substrate concentrations under conditions of oxygen saturation produced linear Lineweaver–Burk plots with highly significant correlation coefficients ($r = 0.990 \pm 0.005$), indicating that linoleic acid hydroperoxidation by LOX-2 and LOX-3 was not subject to allosteric interactions. LOX-2 from *E. coli* and pea seeds had identical k_{cat} values of 2 s^{-1} ; the LOX-3 enzymes had similar values of 65 and 78 s^{-1} (Table 1). Estimates of k_{cat}/K_m for linoleic acid hydroperoxidation of 6.7 $\text{mM}^{-1} \cdot \text{s}^{-1}$ for LOX-2 enzymes and 9.4 and 12.2 $\text{mM}^{-1} \cdot \text{s}^{-1}$ for LOX-3 from *E. coli* and pea seeds, respectively, suggest that the catalytic efficiencies of both isoforms for this reaction were similar. The pH profiles of linoleic acid hydroperoxidation for LOX-2 enzymes were significantly narrower than LOX-3 enzymes (see Figures 3a and 3d), although the optimum pH (approx. 6.3) for the reactions was very similar. Reproducible dips in the pH profile around pH 6.0 have been observed for pea LOX in other studies [8] and may

Figure 2 Interfacial phenomena in pea LOX catalysis: effect of LOX-2 and LOX-3 from *E. coli* and pea seeds on linoleic acid hydroperoxidation in the presence and absence of Tween 20

Reactions (1 ml) of LOX from *E. coli* (solid lines) or pea seeds (dotted lines) containing up to (a) 26 μg of LOX-2 or (b) 40 μg of LOX-3 were assayed for hydroperoxidation at 234 nm with 0.3 mM linoleic acid in 50 mM sodium phosphate buffer (pH 6.3), with (○, ●), and without (□, ■) 0.01% (w/v) Tween 20 at 25 °C. Data for LOX-2 with Tween 20 were fitted by linear regression. Inset in (b) shows the determination of purified LOX-3 specific activity, with the same axis units as (b).

Table 1 Comparison of kinetic parameters for linoleic acid hydroperoxidation by LOX-2 and LOX-3 purified from *E. coli* and pea seeds

All parameters were determined using the standard assay for hydroperoxidation with 0.3 mM linoleic acid and 0.01% (w/v) Tween 20 at pH 6.3 and 25 °C (see Experimental section for details).

Parameter	LOX-2		LOX-3	
	Recombinant	Pea	Recombinant	Pea
Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	1.8	1.2	40	48
K_m (mM)	0.3	0.3	6.9	6.7
V_{max} ($\mu\text{mol}/\text{min}$)	0.01	0.01	0.4	0.3
k_{cat} (s^{-1})	2	2	65	78
k_{cat}/K_m ($\text{mM}^{-1}\cdot\text{s}^{-1}$)	6.7	6.7	9.4	12.2
pH optimum (approx.)	5.8–6.4	5.8–6.4	5.7–6.7	5.6–6.5

relate to ionization changes that affect micellar associations between linoleic acid and detergent, and substrate availability to the fatty acid pocket.

The substrate and product specificities of LOX-2 from *E. coli* and pea seeds and of LOX-3 from *E. coli* and pea seeds were very similar, but those of LOX-2 and LOX-3 from either source were quite different (Table 2). The preferred substrate of both isoforms from *E. coli* and pea seeds for hydroperoxidation, and for carbonyl production by LOX-3, was linoleic acid. For both LOX-2 enzymes, significant activity with its methyl ester and linolenic acid was observed. In contrast, both LOX-3 enzymes had a very narrow substrate specificity and had activity almost exclusively with linoleic acid, which suggested that the fatty acid pocket of LOX-3 enzymes had a less accommodating structure than that of the LOX-2 enzymes.

LOX-3, but not LOX-2, enzymes also exhibited carbonyl production, as detected at 280 nm, from linoleic acid oxidation in the pH range 5.5–9 (spectral changes shown at pH 6.3 only, Figures 4a and 4b), and from the oxidation of linolenic (Figures 4c and 4d) and arachidonic (Figures 4e and 4f) acids at pH 6.3. Carbonyl production from linoleic acid under the standard assay conditions was always less than or equal to the level of hydroperoxide production; the relative proportions were affected by assay pH (Figure 3c) and enzyme concentration in the assay (Figure 5). Under the limited conditions tested (pH 6.3 and single enzyme concentration), this was also true for linolenic and arachidonic acids (Table 2). Carbonyl production from linoleic acid slightly exceeded hydroperoxide production only when the assay pH was increased to 6.5–7.0 (Figure 3c). The progress curves for hydroperoxide production by LOX-2 and LOX-3, and for carbonyl production by LOX-3, were essentially sigmoidal with a lag phase indicative of enzyme activation [49]. This was followed by an extended linear (steady-state) region, then saturation due to substrate exhaustion or self-inactivation, and was typical of other characterized LOXs. The kinetics of methyl linoleate hydroperoxidation by LOX-2 from *E. coli* and pea seeds were non-linear and an initial burst of activity was observed, which slowed rapidly. These kinetics may be related to the presence, albeit at low concentrations, of ethanol (1.5% v/v) and acetone (1% v/v) in the reactions that were required to emulsify the substrate.

The main products of linoleic acid oxidation at pH 6.3 for LOX-2 and LOX-3 from *E. coli* and pea seeds were identical: namely, 13-HPODE and 9-HPODE. Other products included stereoisomers of these compounds and hydroxy acids (Z. Wu, R. K. Hughes, R. Casey and D. S. Robinson, unpublished work). For both LOX-3 enzymes their ratios of production (13-HPODE:9-HPODE, 1:2) were identical with that of a partially purified LOX preparation from a mutant pea line lacking LOX-2 [4]. For both LOX-2 enzymes the ratios were similar (7:1 and 4:1 for LOX-2 from *E. coli* and pea seeds, respectively; Table 2), but different to LOX-3. We believe that the differences in production of 13-HPODE relative to 9-HPODE from linoleic acid by the two LOX-2s, and higher reactivity with arachidonic acid for recombinant LOX-2 compared with that from pea seeds, is related to the fact that pea-seed LOX-2 produced in *E. coli* is a single species, whereas that purified from pea, derived from 2–3 genes [3], may be a mixture of several very similar isoforms [50].

These data indicate that, despite 86% similarity at the amino acid sequence level, LOX-2 and LOX-3 (from *E. coli* and pea seeds) have different positional specificities and are primarily 13- and 9-LOXs, respectively. This may suggest that a mechanism exists to recognize the orientation of linoleic acid in the fatty acid pocket of pea LOX [51] in a manner similar to human 5- and 15-LOXs [52]; differences in reactivity of the two isoforms with methyl linoleate would support this idea. However, spatial restraints dictate that the fatty acid must enter the pocket in an extended conformation and that alignment of the fatty acid in

Figure 3 pH profiles for reactions of LOX-2 and LOX-3 from *E. coli* and pea seeds with linoleic acid

Reactions (1 ml) containing similar activity levels of LOX-3 from *E. coli* (solid lines) or pea seeds (dotted lines) under the standard conditions with 0.3 mM linoleic acid in acetate, Mes, phosphate, Hepes and Tris buffers from pH 4.5 to 9.0 (see Experimental section for details), containing 0.01% (w/v) Tween 20 at 25 °C, were assayed for (a) hydroperoxidation and (b) carbonyl production at 234 nm and 280 nm, respectively. Rates were calculated from the linear region of the progress curves to determine (c), the ratio of carbonyl to hydroperoxide (HPODE; defined as 13-HPODE and 9-HPODE) and hydroxy acid (HODE; defined as 13-HODE and 9-HODE) production. Similar activity levels of LOX-2 were assayed in the same buffers for hydroperoxidation at 234 nm (d).

Table 2 Substrate and product specificities of LOX-2 and LOX-3 from *E. coli* and pea seeds

All activities were determined at pH 6.3 and 25 °C with 0.3 mM substrate and 0.01% (w/v) Tween 20 by increase in A_{234} [hydroperoxide (HPODE; defined as 13-HPODE and 9-HPODE) and hydroxy acid (HODE; defined as 13-HODE and 9-HODE) production] or A_{280} (carbonyl production). Linolenic and arachidonic acids were prepared as for the standard assay. Methyl linoleate was an emulsion in acetone/ethanol/phosphate buffer (1:1.5:97.5, by vol.) [8] (see Experimental section for details).

Property	LOX-2		LOX-3	
	Recombinant	Pea	Recombinant	Pea
Substrate specificity (relative rate of hydroperoxidation, %)				
Linoleic acid	100	100	100	100
Linolenic acid	60	54	5	4
Arachidonic acid	27	2	4	7
Methyl linoleate	90	76	0	0
Product specificity				
Ratio of positional isomers of hydroperoxides (13-HPODE:9-HPODE) from	7:1	4:1	1:2	1:2
linoleic acid				
Carbonyl production	No	No	Yes	Yes
Ratio of carbonyl to HPODE + HODE production				
Linoleic acid	—	—	0–0.5	0–0.5
Linolenic acid	—	—	0.5	0.5
Arachidonic acid	—	—	0.2	0.2

the pocket may be the key determinant of positional specificity [53].

A detailed comparison of the properties of LOX purified from peas in other studies [6–10] with those described in the present

study is difficult. Despite apparent homogeneity and a single pI, pea-seed LOX preparations may contain different LOX activities resulting from only subtle changes in primary structure [5] and be dependent on the pea cultivar. Nevertheless, LOX-2 and LOX-3 purified from pea seeds in the present study were indistinguishable from the recombinant products. In relation to carbonyl production, LOX-2 and LOX-3 bore some similarities with pea LOX PLII and LOX PLI, respectively, described elsewhere [7,8], and with the LOX-2 and LOX-1, respectively, described by Sanz et al. [10]. The product specificity of LOX-3 in the present study also showed marked similarity with pea LOX-1 described by Kuhn et al. [6]. In agreement with LOX-2 and LOX-3 in the present work, pea LOX PLI and PLII [8] similarly showed large differences in reactivity with methyl linoleate. Activity of LOX-2 enzymes with esterified fatty acids in the present work was typical of other LOXs, which show high relative activities with linolenic acid [28].

Recombinant LOX-2 and LOX-3 were both purified as the EPR silent Fe(II) forms. This is true of most plant LOXs [23], but they can be isolated in the oxidized Fe(III) state, and a small proportion of iron in all active LOX must exist in this state. A signal at g_6 could be obtained for both recombinant isoforms after activation with 9-HPODE (Figure 6) or 13-HPODE (results not shown), and suggested the production of Fe(III) LOX from Fe(II) LOX in the catalytic cycle. The g_6 peak arises from part of the $S = 5/2 | \pm 1/2 \rangle$ Fe(III) centre signal ($| \rangle$ is defined as a ket), which extends to g_2 (results not shown). This notation indicates that we are observing the $\pm \frac{1}{2}$ Kramer's doublet of the $S = 5/2$ spin state. At g_2 the weak $S = 5/2$ signal overlaps with Mn(II) signals and a free radical signal. The shape of the g_6 signal indicates that axial and near-axial components are present.

Figure 4 Spectral changes during reaction of recombinant LOX-2 and LOX-3 with different fatty acids

Reactions (1 ml) containing similar activity levels of (a,c,e) LOX-2 or (b,d,f) LOX-3 under the standard conditions (see Experimental section for details), with 0.3 mM linoleic acid (a,b), linolenic acid (c,d) or arachidonic acid (e,f) in 50 mM sodium phosphate buffer (pH 6.3) containing 0.01% (w/v) Tween 20, were scanned at 50 s intervals from 200 to 350 nm at 25 °C in a Shimadzu 2401-PC UV/VIS spectrophotometer. Behaviour of LOX from pea seeds was indistinguishable from the above.

Figure 5 Linoleic acid oxidation products from recombinant LOX-3

Reactions (1 ml) containing up to 14 μg of recombinant LOX-3, with 0.3 mM linoleic acid in 50 mM sodium phosphate buffer (pH 6.3) containing 0.01% (w/v) Tween 20 at 25 °C, were assayed for (a) hydroperoxidation (solid line) and carbonyl production (dotted line) at 234 nm and 280 nm, respectively. (b) Shows the ratio of carbonyl to hydroperoxide (HPODE; defined as 13-HPODE and 9-HPODE) and hydroxy acid (HODE; defined as 13-HODE and 9-HODE) production calculated from (a) and fitted to the function $y = 1 - e^{-x}$.

Similar heterogeneity was observed for soya bean LOX-1 [54] and human 5-LOX [55]. Weak signals were detected at $g_{4.3}$ before treatment with HPODE, presumably due to adventitious

iron. After HPODE treatment the $g_{4.3}$ peak sharpened and increased in concentration. This peak has been attributed to $S = 5/2 | \pm 3/2 > \text{Fe(II)}$ centres, again several forms of the iron centre appear to be present. The conditions for generation of recombinant Fe(III) LOX-2 and Fe(III) LOX-3 were different, and the enzymes required prolonged (10 min) and rapid (approx. 20 s) reactions respectively. The 30-fold difference in time required for development of the g_6 signal for the two isoforms was supported by a 36-fold difference in k_{cat} determined for linoleic acid hydroperoxidation.

The specific activity of both LOX-3 enzymes increased during the purification to apparent homogeneity and the total iron content of purified recombinant LOX-3 was approximately 1 mol/mol of enzyme. Only a minority of the iron (approx. 10%) was EPR-detectable, probably due to the non-optimization of EPR conditions. The specific activity of LOX-2 from both sources increased and then decreased during the purification to apparent homogeneity and suggested that LOX-2 was a less stable structure than LOX-3. This has been confirmed by thermal stability studies on the two enzymes (M. D. Busto, R. K. Owusu-Apenten, D. S. Robinson, Z. Wu, R. Casey and R. K. Hughes, unpublished work). Nevertheless, the biochemical properties of the partially purified (results not shown) and purified enzymes from both sources were indistinguishable. Despite the very low proportion of EPR-detectable iron in purified recombinant LOX-2 (approx. 1%), total iron analysis suggested that it was not iron-deficient (approx. 2 mol/mol of enzyme). This higher level of iron was probably related to the absence of EDTA during the purification, due to concerns over stability and the potential loss of iron. This suggested that differences in the biochemical properties of LOX-2 and LOX-3 were not related to loss of iron but were related to differences in enzyme structure and/or mechanism.

Figure 6 EPR spectroscopy of hydroperoxide-activated recombinant pea LOX-2 and LOX-3

Spectra were determined before and after hydroperoxide activation of (a) LOX-2 or (b) LOX-3. Prior to freezing in liquid nitrogen, LOX-3 (200 μ M) was incubated at room temperature for about 20 s with a molar equivalent of 13-HPODE. Incubation of LOX-2 (100 μ M) under the same conditions as LOX-3 gave no $g6$ signal; treatment with another molar equivalent of 13-HPODE for 10 min at room temperature produced the weak signal shown at $g6$. Shorter or longer incubations did not improve signal intensity. Background $g6$ signals from buffer (50 mM sodium phosphate buffer, pH 6.3) controls and resting enzymes were negligible (see Experimental section for details).

Reaction mechanisms

The reaction mechanisms of LOX-2 and LOX-3 with linoleic acid seem to be different. LOX-2 and LOX-3 produced the same hydroperoxides, but in different ratios, under the same aerobic assay conditions, yet only LOX-3 produced carbonyl compounds. This demonstrated that carbonyl production was a truly aerobic reaction of LOX, was a characteristic feature dependent on LOX structure and was not merely a response of LOX to anaerobiosis [25]. It was most unlikely that the difference in reaction products resulted from differences in K_m for oxygen ($< 2.5 \mu$ M for soya bean LOX-1; [56]); carbonyl production was observed early (< 30 s) during the aerobic reaction of LOX-3 with linoleic acid, where oxygen concentration was close to saturation (258 μ M at 25 $^{\circ}$ C; [56]). Analysis of the crystal structures of soya bean LOX-1 and LOX-3 indicates that oxygen could permeate through the LOX molecule [31,32].

We suggest that release of the peroxy radical formed from the reaction of the fatty acid radical with oxygen is the likely, non-enzymic, source of the vast majority of the carbonyls. All the carbonyls in our reactions were ketodienoic acids with equimolar amounts of hydroxy acids (Z. Wu, R. K. Hughes, R. Casey and D. S. Robinson, unpublished work). This suggests that a simple dismutation of two peroxy radicals to yield a ketone (producing ketodienoic acid) and an alcohol (producing hydroxy acid), would account for carbonyl production from linoleic acid by

LOX-3 [57,58]. Singlet oxygen, $O_2 (^1\Delta_g)$, would also be produced [58]:

Our scheme would suggest that rate of release of the peroxy radical rather than the fatty acid radical is the key determinant of carbonyl production and that both radicals remain bound in LOX-2. A proportion of the peroxy radical would have to remain enzyme-bound in both isoforms in order to regenerate hydroperoxide in the catalytic cycle and maintain LOX-re-activation (Scheme 1). The fatty acid radical is unlikely to be released, as it has extremely high reactivity with oxygen and $\text{LOO}^{\bullet}$ would be formed very quickly. This agrees with studies on soya bean LOX-1, which suggest that the fatty acid radical remains bound in the presence of oxygen [24].

Hydroperoxidase activity has been widely used to describe carbonyl production:

Where LOOH is hydroperoxide, $\text{L}^{\bullet}$ is the fatty acid radical, L-OH is hydroxy acid, $\text{LOO}^{\bullet}$ is the peroxy radical and LH is linoleic acid. The reaction of hydroperoxide and linoleic acid would yield fatty acid radical, hydroxy acid and water (i); further reaction of fatty acid radical with oxygen would yield a peroxy radical (ii), which could then react with more linoleic acid to regenerate hydroperoxide and fatty acid radical (iii). Hydroperoxidase activity in combination with the rate of release of peroxy radical can be used to interpret the differences in reaction mechanism of LOX-2 and LOX-3. Thus, in LOX-3, the majority of the peroxy radical produced in reaction (ii) is released for carbonyl production, whereas in LOX-2 this radical remains bound for reaction (iii); no carbonyls are produced. Perhaps the much higher affinity of LOX-2 than LOX-3 for linoleic acid accounts for this fact; the rate of reaction (iii) in LOX-2 would be so much faster than in LOX-3 and peroxy radical would not be released and dismutated to carbonyls. Differences in the reaction rates of (i) and (iii) are likely to determine the relative proportions of hydroperoxide and hydroxy acid.

In the present work, at very low enzyme concentrations no carbonyls were produced, but increasing enzyme concentration resulted in the production of large amounts of carbonyls. This suggested that the regeneration of hydroperoxide, which was required for cycling between Fe(II) and Fe(III) LOX to reactivate LOX in the catalytic cycle, was a prerequisite that preceded carbonyl production. The effect of increasing enzyme concentration may have been to allow production of fatty acid radical and hydroperoxides at a level above that required for reactivation; the excess would participate in carbonyl production by formation of peroxy radical which was dismutated.

Differences in the capacities of LOX for carbonyl production in the presence of fatty acid and oxygen clearly suggest that LOX may not share a common mechanism for this reaction. We suggest that radicals can dissociate more readily from LOX to participate in secondary reactions than predicted from studies on soya bean LOX-1, with oxygen playing a major, but different role, in LOX catalysis. Exactly what elements of LOX structure determine the fate of these radicals remains to be elucidated. The peroxy radical would appear to be of prime importance in other

Scheme 1 Catalytic cycles of linoleic acid hydroperoxidation and carbonyl production for pea-seed LOX-2 and LOX-3

Reaction schemes are based on those for soya bean LOX-1 [24]. LOX activation by hydroperoxide (LOOH) is followed by hydrogen abstraction from linoleic acid (LH) producing fatty acid radical (L[•]) and recycling to regenerate hydroperoxide. Carbonyl production by LOX-3 starts by reaction of L[•] with oxygen to form a peroxy radical (LOO[•]). LOO[•] is probably released in preference to L[•]. Two peroxy radicals oxidize Fe(II) LOX-3 to form Fe(III) LOX-3 and undergo a dismutation reaction to yield equimolar amounts of a carbonyl and an alcohol, which produce the ketodiene and hydroxy acid, respectively, and oxygen. In LOX-2, L[•] and LOO[•] are retained and no carbonyls or hydroxy acids are produced.

LOX secondary reactions and may be significant in the formation of co-oxidation products (Z. Wu, R. K. Hughes, R. Casey and D. S. Robinson, unpublished work).

The present work represents the first characterization of single species of the two major isoforms of LOX in pea seeds and has demonstrated that they have quite distinct properties and reaction mechanisms. We have established carbonyl production as a true aerobic reaction dependent on LOX structure and have provided new evidence for a carbonyl production mechanism. Expression in *E. coli* of pea LOX has made possible the large-scale production of recombinant wild-type enzymes and the engineering and characterization of rationally designed mutants or hybrids for the application of biophysical, kinetic and modelling techniques. These will be used to further probe the primary determinants of carbonyl production and the precise roles of the fatty acid and peroxy radicals and oxygen in this reaction. An investigation of the commercial potential of authentic recombinant LOX-2 and LOX-3 to modify bread-making processes and produce novel natural flavour compounds is now possible.

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