Probing a novel potato lipoxygenase with dual positional specificity reveals primary determinants of substrate binding and requirements for a surface hydrophobic loop and has implications for the role of lipoxygenases in tubers

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A new potato tuber lipoxygenase full-length cDNA sequence (*lox1:St:2*) has been isolated from potato tubers and used to express in *Escherichia coli* and characterize a novel recombinant lipoxygenase (potato 13/9-lipoxygenase). Like most plant lipoxygenases it produced carbonyl compounds from linoleate (the preferred substrate) and was purified in the Fe(II) (ferrous) state. Typical of other potato tuber lipoxygenases, it produced 5-HPETE [5(*S*)-hydroperoxy-(6*E*, 8*Z*, 11*Z*, 14*Z*)-eicosatetraenoic acid] from arachidonate. In contrast to any other potato tuber lipoxygenase, it exhibited dual positional specificity and produced roughly equimolar amounts of 13- and 9-hydroperoxides (or only a slight molar excess of 9-hydroperoxides) from linoleate. We have used a homology model of pea 9/13-lipoxygenase to superimpose and compare the linoleate-binding pockets of different potato lipoxygenases of known positional specificity. We then tested this model by using site-directed mutagenesis to

identify some primary determinants of linoleate binding to potato 13/9-lipoxygenase and concluded that the mechanism determining positional specificity described for a cucumber lipoxygenase does not apply to potato 13/9-lipoxygenase. This supports our previous studies on pea seed lipoxygenases for the role of pocket volume rather than inverse orientation as a determinant of dual positional specificity in plant lipoxygenases. We have also used deletion mutagenesis to identify a critical role in catalysis for a surface hydrophobic loop in potato 13/9-lipoxygenase and speculate that this may control substrate access. Although potato 13/9-lipoxygenase represents only a minor isoform in tubers, such evidence for a single lipoxygenase species with dual positional specificity in tubers has implications for the proposed role of potato lipoxygenases in the plant.

Key words: modelling, mutagenesis.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12, linoleate: oxygen oxidoreductase, LOX) refers to a broad class of non-haem iron-containing dioxygenases that catalyse the oxidation of polyunsaturated fatty acids containing a 1-*cis*, 4-*cis*-pentadiene system, such as linoleate, to produce conjugated unsaturated fatty acid hydroperoxides [1,2]. Manganese-containing LOX has also been described [3]. Although details of the LOX reaction mechanism(s) are still to be resolved, it is clear that positional specificity, which refers to the carbon atom on the fatty acid chain at which there is insertion of molecular oxygen, is highly variable amongst plant and mammalian LOXs. Aspecificity of oxygen insertion, despite specificity in hydrogen abstraction from the substrate, means that some plant LOXs produce both 9-HPODE [9-hydroperoxy-(10*E*, 12*Z*)-octadeca-10, 12-dienoic acid] and 13-HPODE [13-hydroperoxy-(9*Z*, 11*E*)-octadeca-9, 11-dienoic acid] from linoleate and are said to exhibit dual positional specificity [4]. LOX heterogeneity is widespread, and LOXs purified from the native source probably contain a number of different isoforms with highly similar primary sequences and isoelectric points but different specificities [1]. Dual positional specificity of LOX

purified from the native source can therefore be interpreted as a consequence of the analysis of heterogeneous mixtures. From studies of recombinant LOX expressed in *Escherichia coli*, where the protein is expressed from a single cDNA sequence and LOXs have not been detected in prokaryotes [1], it is clear that single LOX species can exhibit dual positional specificity (under normal aerobic conditions). The characterization of recombinant LOX is therefore gaining importance for studying the molecular basis of dual positional specificity and for many other aspects of LOX catalysis, including carbonyl production and co-oxidation behaviour. In order to facilitate the correlation of a particular cDNA sequence with enzymic properties, recombinant plant LOXs have, like mammalian LOXs, been reclassified on the basis of positional specificity [4].

In the plant, the activity of LOX on polyunsaturated fatty acids generates hydroperoxide products which act as substrates for other enzymes such as allene oxide synthase [5] and hydroperoxide lyase [6]. The products of these enzyme reactions, oxylipins (oxidized fatty acid derivatives), can act as elicitors of defence gene expression and as anti-microbial compounds [7]. The characterization of recombinant LOX produced in a heterologous expression system can help to clarify a particular role for

Abbreviations used: LOX, lipoxygenase; 13-HPODE, 13-hydroperoxy-(9*Z*, 11*E*)-octadeca-9, 11-dienoic acid; 9-HPODE, 9-hydroperoxy-(10*E*, 12*Z*)-octadeca-10, 12-dienoic acid; 5-HPETE, 5(*S*)-hydroperoxy-(6*E*, 8*Z*, 11*Z*, 14*Z*)-eicosatetraenoic acid; Compound I, (5*S*, 12*R*)-dihydroxy-(6*E*, 8*E*, 10*E*, 14*Z*)-eicosatetraenoic acid; Compound II, (5*S*, 12*S*)-dihydroxy-(6*E*, 8*E*, 10*E*, 14*Z*)-eicosatetraenoic acid; c.m.c., critical micellar concentration.

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The nucleotide sequence of *lox1:St:2* encoding potato tuber 13/9-lipoxygenase will appear in the GenBank[®] and EMBL databases under accession number Y18548.

LOX in the plant [8]. Identifying those LOX transcripts that code for active proteins can be determined by expressing them in *E. coli*. Subsequent evaluation of their product, including positional specificity, will provide an understanding of the nature of the oxylipin products to which they may contribute *in planta*.

Potato tuber extracts contain numerous LOXs that can be separated by anion-exchange chromatography, and many different isoforms have been purified from different varieties of potato tuber. The major form appears consistently to be a typical 9-LOX or 5-LOX, assayed with linoleate or arachidonate, respectively [9,10]. Crystals of a major native potato tuber LOX have been obtained but the structure has not been resolved [11]. A number of workers have also isolated LOX cDNA sequences from leaves, roots and tubers of potatoes and expressed recombinant LOX from them in *E. coli* [12,13]. Some have been characterized partially and are of known positional specificity. Others have isolated full-length cDNA sequences from potatoes, but it is not known if they correspond to active proteins [14,15]; potato tuber LOX sequences submitted to the GenBank® and EMBL databases under accession numbers U60200 and U60201. The substrate and positional specificity of recombinant LOXs from tubers and leaves have been shown to be different [12]. Potato 9-LOX (T8) genes were expressed mainly in tubers (weakly in roots), potato 13-LOX1 (H1) in leaves and potato 13-LOX2 (H3) in leaves and roots (and weakly in tubers) [12]. The 9- and 13-LOXs preferred linoleate (abundant in tubers) and linolenate (abundant in leaves) as substrate, respectively. Not all these LOX isoforms have a role in the synthesis of the plant hormone jasmonate (via allene oxide synthase from linolenate). Jasmonate has a role in plant protection against insect pests, and is produced on insect damage whereupon it activates proteinase-inhibitor genes [7,16], but its synthesis requires 13-(S)-hydroperoxy-octadecatrienoic acid (13-HPOTE) as a precursor, which suggests that 9-LOXs have no role in this pathway. However, the transcript corresponding to potato 13-LOX2 was detected weakly in tubers [12], which suggested that the production of 13-HPOTE in tubers by a 13-LOX was possible. 9-HPOTE has also been shown to be the major hydroperoxide product of LOX in potato leaves [17]. No study has found LOX in potatoes (leaves or tubers) that exhibit dual positional specificity with linoleate as substrate, with both 9- and 13-LOX activities residing in a single protein.

Potato tuber LOXs are of interest to the fine chemicals and pharmaceutical industries because of their ability to catalyse the first committed step in the synthesis of leukotrienes and lipoxins, which are potent elicitors of allergies and other hypersensitivity reactions [18,19]. Potato tuber LOXs have also been shown to have good co-oxidation potential [20].

The primary determinants of positional specificity in mammalian and an atypical plant [21] LOX have been identified. Crystal structures of LOX in the absence of substrate [22,23] are perhaps more unlikely for some arachidonate 5-LOXs since they are known to be relatively unstable when highly purified [24]; none are available in the presence of substrate. In the absence of crystal data, homology modelling and information from site-directed mutants have become increasingly important. The primary sequences of mammalian LOXs bear poor similarity and are larger than those of plant LOXs, but there is good evidence that their domain structures are similar [25]. Many plant LOX sequences are available and some are highly similar and of known positional specificity [4]. It has proved possible to identify important roles for equivalent residues in plant LOXs to those which affect catalysis in mammalian LOXs simply from sequence comparisons, active-site modelling and the characterization of site-directed mutants [22,26].

There is a need to identify primary determinants of catalytic behaviour in plant LOX. This is best achieved by the modelling and characterization of LOX that have very similar primary sequences but different properties and specificities and by the construction and characterization of site-directed mutants. We sought therefore to: express and characterize a recombinant potato tuber LOX (a number of potato LOX primary sequences were available); produce a homology model of linoleate binding to this enzyme based on another described for pea 9/13-LOX (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work); validate this model by the construction and characterization of site-directed mutants; assess the relevance of pocket volume and inverse orientation theories for plant LOXs with dual positional specificity; and relate the properties of the potato LOX to its potential role in the plant. We were also interested in the role of a hydrophobic loop modelled on the surface of this potato LOX, and the effect of removing it was determined by deletion mutagenesis.

EXPERIMENTAL

Materials

Oligonucleotides were obtained from Genosys (Cambridge, U.K.). Glycerol stocks of *E. coli* strains were obtained from Novagen (Madison, WI, U.S.A.). Bacterial growth media were as described in [27]. Kanamycin and LOX substrates were from Sigma (Poole, Dorset, U.K.). 5(S)-Hydroperoxy-(6E, 8Z, 11Z, 14Z)-eicosatetraenoic acid (5-HPETE), (5S, 12R)-dihydroxy-(6E, 8E, 10E, 14Z)-eicosatetraenoic acid (Compound I) and (5S, 12S)-dihydroxy-(6E, 8E, 10E, 14Z)-eicosatetraenoic acid (Compound II) were from Cascade Biochemistry (Reading, Berks., U.K.). A sample of recombinant potato (*Solanum tuberosum* cv. Desiree) tuber LOX (potato 9/13-LOX) [13] was obtained from Professor Channa Reddy (Pennsylvania State University, University Park, PA, U.S.A.).

cDNA cloning

Poly(A)⁺-containing RNA (90 µg) was prepared from total RNA from freeze-dried potato (*S. tuberosum* cv. Desiree) tubers (44 g) and size-fractionated on denaturing sucrose gradients [28]. RNA was recovered from gradient fractions by ethanol precipitation and analysed by Northern blotting and hybridization [29], using a 1.1 kb *EcoRI* fragment of the potato tuber LOX cDNA clone *lox1:St:1* [15]. Fractions containing RNA of approx. 3 kb that hybridized to the *lox1:St:1* probe were pooled, precipitated and 4 µg used to generate a small cDNA library in pUC18. Double-stranded, blunt-ended cDNA was produced using a cDNA synthesis kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, St. Albans, Herts., U.K.) and ligated to a 2-fold molar excess of *SmaI*-cut, phosphatase-treated pUC18 (Amersham Pharmacia Biotech). The ligated DNA was transformed into XL10 cells (Stratagene, Cambridge, U.K.), plated on to Luria-Bertani plates containing ampicillin, X-gal and isopropyl β-D-thiogalactoside and 800 colonies screened by colony hybridization [30] using the 1.1 kb *EcoRI* probe (see above). Positive clones were assessed for insert size by PCR, using M13 universal and reverse primers, and those containing inserts of approx. 2.8 kb were examined further by DNA sequencing. The cDNA clone used for expression of potato 13/9-LOX in *E. coli* was referred to as pUCLOX53.

Cloning for expression of wild-type potato 13/9-LOX

Full-length tuber LOX cDNA in pUCLOX53 was transferred to pSBETa, a derivative of pET3a that contains the *argU* gene

coding for rare tRNA_{Arg4} [31]. This prevents mistranslation at consecutive AGG and AGA codons that have rare usage in *E. coli* and has been described for the expression of other recombinant proteins [32]. LOX cDNA was amplified by PCR from pUCLOX53 using *Pfu* polymerase (Stratagene) with *Nde*I and *Bam*HI restriction sites introduced at the 5' and 3' ends of the gene, respectively. The product was partially digested with *Nde*I and *Bam*HI, gel-purified by electroelution and inserted into *Nde*I/*Bam*HI-digested pSBETa. As part of the PCR amplification of the *Nde*I/*Bam*HI fragment, the codon preference for the first nine amino acids of LOX was optimized [33] by changing the codons for amino acids 8 and 9 from GGACTA to GGCCTG. The termination codon was also changed from TAG to TAA. This final construct was referred to as pSBPOTLOX2. DNA sequencing was carried out using an ABI sequencer (Perkin Elmer Applied Biosystems, Warrington, Cheshire, U.K.) and a thermosequenase dye terminator cycle-sequencing kit (Amersham Pharmacia Biotech), both as recommended by the manufacturers. Both strands of the LOX coding sequence in pSBPOTLOX2 in the *E. coli* expression strain BL21(DE3) were sequenced.

Mutant construction

Mutations were introduced into pSBPOTLOX2 plasmid DNA purified from *E. coli* BL21(DE3) cells using the QuikChange site-directed mutagenesis kit and *Pfu* polymerase (Stratagene). Mutant expression constructs were referred to as pSBN1 ($\Delta 250 \rightarrow 254$ mutant), pSBO12 (I437L mutant), pSBP1 (V570I mutant) and pSBQ2 (V580F mutant) and both strands of the LOX coding sequence in these constructs were determined to confirm the absence of any unwanted mutations.

LOX expression and detection

Wild-type or mutant expression constructs were transferred into *E. coli* BL21(DE3) cells for protein expression as for pea seed LOX [27], except the cultures were incubated at 15–18 °C. No isopropyl β -D-thiogalactoside was required for expression. LOX polypeptides were detected by SDS/PAGE and immunoblotting [27] using an antibody (anti-B) to pea seed LOX [34]. The level of expression of mutant compared with wild-type LOX was determined from protein blots of *E. coli* extracts containing the same amount of soluble proteins, highlighting full-length LOX immunoreactive polypeptides on the enlarged gel image using the 'trace contour' function in the software program Adobe Photoshop 5.0, and weighing the paper cutouts.

Protein purification

All manipulations were carried out at 4 °C or on ice. Cultures were centrifuged at 5000 *g* for 5 min and cells were homogenized in 25 mM Bis-Tris buffer, pH 7.1 (iminodiacetic acid), containing 1 mM EDTA and 1 mM dithiothreitol (buffer A). Protease inhibitors were also included in the extraction buffer using a protease-inhibitor cocktail (Sigma product number P2714) that consisted of 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), bestatin, leupeptin and aprotinin. This was supplemented further with 1 μ M pepstatin A, 10 μ M chymostatin and 0.5 mM PMSF. The extract was then passed twice through a French Press (1500 p.s.i., or 10350 kPa) and centrifuged at 10000 *g* for 10 min. The LOX-containing supernatant was purified by chromatofocusing on a Polybuffer Exchanger 94 column (1.6 $\times$ 19 cm) equilibrated with buffer A. Unbound material was discarded and bound material eluted with 10% (v/v) Poly-

buffer 74, pH 3.5. Fractions containing LOX activity were pooled, adjusted to pH 6.1 (1 M Bis-Tris buffer, pH 7.1) and concentrated by ammonium sulphate precipitation (60% saturation) followed by centrifugation at 20000 *g* for 30 min. The pellet was resuspended in 50 mM sodium phosphate buffer, pH 6.8, centrifuged at 12000 *g* for 5 min to remove insoluble protein, and concentrated by ultrafiltration (Centriplus-50 and Centricon-50; Millipore, Watford, Herts., U.K.). Further purification was achieved on Superdex 200 and Mono-Q [27]. Enzyme was desalted on Sephadex G-25 into 25 mM Bis-Tris buffer, pH 7.1, concentrated in a Centricon-50 to ≥ 10 mg/ml and stored at -70 °C in small aliquots after flash-freezing in liquid nitrogen. These storage conditions were important, as this enzyme, like some other purified potato tuber LOX, was found to be quite unstable and lost, for example, 40% activity overnight at 4 °C as a suspension in ammonium sulphate. The partially purified enzyme was considerably more stable. Protein concentration was estimated as described in [27].

Mutant LOX were partially purified in the same way as the wild type, but the crude *E. coli* extracts were first purified on Q-Sepharose (Amersham Pharmacia Biotech). The column was equilibrated with 20 mM Bis-Tris buffer, pH 6.5, containing 1 mM EDTA and bound protein eluted with a 0–0.5 M NaCl gradient. LOX-containing fractions were pooled, desalted and reloaded on a new Q-Sepharose column and eluted with a 0–0.3 M salt gradient in the same buffer. LOX-containing fractions were pooled and purified further as for the wild-type on MonoQ and Superdex 200 [27].

Determination of kinetic properties and substrate/product specificity

Substrate solubilization, kinetic parameters and substrate and product specificities were measured as for recombinant pea LOX [27]. Positional specificity was calculated as the ratio of formation of 13-Z, *E*-HPODE/9-*E*,Z-HPODE [35].

Arachidonate oxidation

Arachidonate was solubilized in ethanol and reaction mixtures [36] were acidified and extracted with diethyl ether, dried with sodium sulphate and the residue redissolved in hexane for HPLC purification. Hydroperoxide and hydroxyacid products were detected at 234 nm on a Zorbax 7 μ m silica (250 $\times$ 4.6 mm) column [35] and identified from the retention times of authentic standards [5-, 8-, 12- and 15-hydro(pero)xyeicosatetraenoic acids]. Attempts were made to identify leukotriene hydrolysis products (Compounds I and II) detected at 270 nm on a Nucleosil C₁₈ 3 μ m, 120 Å column (150 $\times$ 4.6 mm) eluted with methanol/water/acetate (70:30:1), pH 5.6 (NH₄OH) [37], also from the retention time of authentic standards. Chromatograms of products arising non-enzymically were produced from reactions in the absence of LOX and the proportion of products due to LOX was calculated by difference.

Preparation of crude LOX extracts from tubers

For positional specificity measurements, tubers (100 g) were homogenized for 30 s with 50 mM sodium phosphate buffer, pH 6.3 (100 ml), containing 2 mM citrate and 2 mM sodium metabisulphite, filtered through muslin and centrifuged at 25000 *g* for 45 min. The supernatant was defined as the crude potato tuber LOX extract. For isoelectric focusing gels, 1 g of freeze-dried potato tuber was mixed with 1 g of polyvinylpyrrolidone and blended on ice in 10 ml of 150 mM sodium phosphate buffer, pH 7.0, containing 5 mM dithiothreitol. After

centrifugation (14000 *g*, 10 min, 4 °C), ammonium sulphate fractionation (20–80 % saturation at 4 °C) was used to produce 12 mg of a crude protein preparation, which was dissolved in and dialysed against 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol, at 4 °C and stored at 5 mg·ml⁻¹ in small portions at -20 °C.

Isoelectric focusing

Protein was extracted as for crude LOX extracts from tubers, mixed on ice with an equal volume of 40 % (w/v) glycerol containing 2 % (w/v) wide-range Ampholines, and 100 µg of total tuber protein or 40 µg of purified recombinant wild-type potato 13/9-LOX were loaded, each in 40 µl. Potato 13/9-LOX was separated as native enzyme by vertical slab (20 × 19 × 0.1 cm) isoelectric focusing on 7.5 % acrylamide gels (acrylamide/bisacrylamide, 19:1, w/w) containing 10 % (w/v) glycerol and 2 % (w/v) wide-range (pH 3.5–10) Ampholines. The gel was prechilled for 3 h, prefocused (15 min at 200 V, 30 min at 300 V, 1 h at 400 V) and run (20 h at 400 V), all at 4 °C. The catholyte was 25 mM NaOH and the anolyte 20 mM acetic acid. pH gradients were assessed after diffusion of Ampholines from 1 cm segments of a blank gel track into 1 ml of degassed water. Gels were fixed for staining for 30 min in 10 % trichloroacetic acid, washed for 2 h in 1 % trichloroacetic acid and for 1 h in 50 % (v/v) ethanol containing 10 % (v/v) acetic acid, stained for 1 h in 0.025 % Coomassie Brilliant Blue in 10 % acetic acid and destained in 10 % acetic acid. For antibody detection, gels were blotted on to nitrocellulose [27] and developed with anti-B antibody to pea seed LOX [34].

Validation of method for determination of LOX positional specificity using crude extracts

As purification of wild-type and mutant potato 13/9-LOX to homogeneity was not possible due to apparent problems with protein instability on chromatography columns and isoelectric-focusing gels, validation of the method to determine LOX positional specificity using crude *E. coli* extracts was required. Known amounts of 13- and 9-HPODE were incubated in the presence and absence of an extract from a culture of *E. coli* that was transformed with the expression vector (pSBETa) only, and which contained similar amounts of soluble protein as that used to determine positional specificity of wild-type potato 13/9-LOX. Cultures were grown, and reaction conditions were the same as those used to express active LOX and to determine positional specificity. Assays were carried out in triplicate.

EPR spectroscopy

The catalytic-iron environment of wild-type potato 13/9-LOX was probed by EPR spectroscopy of the resting enzyme and after activation with 9-HPODE, prepared from reaction of linoleate with purified tomato 9-LOX. EPR spectra were recorded using a Bruker ER200D-SRC X-band EPR spectrometer equipped with an Oxford Instruments ER900 liquid helium cryostat. All spectra were recorded at 10 K, 10 mW and 10 gauss (1.0 mT) modulation amplitude. Experimental conditions are given in the legend to Figure 7 (see below).

RESULTS AND DISCUSSION

Cloning

A new potato tuber LOX cDNA sequence (designated *lox1:St:2*, GenBank® and EMBL accession number Y18548) was identified from screening of the cDNA library. The nucleotide sequence,

Figure 1 Phylogenetic tree and primary sequence comparisons of potato and selected plant LOXs

The tree was produced using the program PHYLO_WIN. Full-length cDNA sequences coding for LOX polypeptides with unknown activity or positional specificity are identified from their GenBank® and EMBL accession numbers. LOXs of known positional specificity are identified by their new designation [4] and their primary sequences were compared using the program GAP. Percentage similarities and identities (in parentheses) between the paired sequences are indicated.

and hence predicted amino acid sequence, of the LOX insert in the expression clone pSBPOTLOX2 was found to be identical to that of the cDNA clone (pUCLOX53), apart from the intended changes at the 5' end of the gene and the termination codon.

Multiplicity and relative abundance of potato tuber LOX sequences

The *lox1:St:2* sequence adds to a large number of full-length LOX cDNA sequences from potato tubers (Figure 1). The predicted amino acid sequence of *lox1:St:2* differed by just 18 nucleotides and 7 amino acids from *lox1:St:1* [15]. Two other full-length LOX cDNA clones were identified from the library screen (L326 and L620; R. Casey and R. K. Hughes, unpublished work); an analysis of their 5' and 3' coding sequences indicated that L326 was more closely related (but not identical) to potato 9-LOX (T8) [12] and L620 was identical to *lox1:St:1* [15].

Figure 2 Gel electrophoresis and protein blot analysis of wild-type potato 13/9-LOX

(A) Purified wild-type potato 13/9-LOX was run on an SDS/PAGE gel and stained with Coomassie Brilliant Blue (lane II), or blotted and probed with anti-B (lane III). The positions of immunoreactive (solid arrows) and non-immunoreactive (dotted arrows) polypeptides are shown in lane II. Lane I, positions of marker proteins. (B) Wild-type potato 13/9-LOX and wild-type pea 9/13-LOX were run separately (lanes II and III respectively) or together (lane I) on an SDS/PAGE gel, blotted and probed with anti-B. The position of the 97 kDa marker protein is indicated. (C) Isoelectric-focusing gel of potato tuber extract (lane I) and purified recombinant wild-type potato 13/9-LOX (lane II), blotted and probed with anti-B. The position of potato 13/9-LOX is shown.

Mutant constructs

Sequencing of the entire potato 13/9-LOX coding sequence in the mutant expression constructs in the expression strain confirmed the presence of only the desired mutation and no others. The mutations in the present study were confirmed as follows, with changes in the nucleotide sequence underlined: I437L (ATT → CTT), V570I (GTC → ATC) and V580F (GTT → TTT). The nucleotide sequence deleted in the deletion mutant ($\Delta 250 \rightarrow 254$) is underlined: CCTAAAAGTGAAAGCAGGATTCTCTT-ATTCTGAGCTTAGACATCTATGTACCG.

Expression

Wild-type potato 13/9-LOX was expressed as a soluble, active non-fusion protein in *E. coli*, but only by co-expression of rare tRNA_{Arg4} on the same plasmid. That rare tRNA_{Arg4} was required for active LOX expression was puzzling. There were no more consecutive rare arginine codons in this sequence than in pea 13/9-LOX and pea 9/13-LOX, yet these were expressed as soluble and active proteins without a requirement for tRNA_{Arg4} [27]. Other potato tuber LOXs have also been expressed without apparent difficulty both as N-terminal fusions [12] and as non-fusions [13].

Table 1 Kinetic parameters for linoleate hydroperoxidation by wild-type potato 13/9-LOX

All parameters were determined at 234 nm with 0.3 mM linoleate and 0.01% (v/v) Tween 20 in 50 mM Mes buffer, pH 5.5, at 25 °C [27]. Specific activity was calculated from that of the enzyme with 50% purity (peak specific activity during purification).

Parameter	Value
Specific activity	65 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein ⁻¹
K_m (linoleate)	0.2 mM
V_{max}	0.4 $\mu\text{mol} \cdot \text{min}^{-1}$
k_{cat}	106 s ⁻¹
k_{cat}/K_m	530 mM ⁻¹ · s ⁻¹
pH optimum	5.5

Figure 3 Calculation of specific activity of purified wild-type potato 13/9-LOX

The rate of linoleate hydroperoxidation at 234 nm was determined for different amounts of LOX protein in the assay. (Inset) The specific activity was calculated by extrapolation to zero enzyme concentration. The estimate for specific activity of 68 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ for the purified enzyme was calculated by assuming the preparation was 50% pure.

Purification and characterization

Purified wild-type potato 13/9-LOX appeared as a major polypeptide on SDS/PAGE gels with a molecular mass of approx. 97 kDa (Figure 2A), but there was some evidence of proteolysis of the enzyme as with recombinant pea seed LOX [27]. An impurity of approx. 100 kDa, slightly larger than pea LOX-3 (from pea seeds), which did not cross-react with anti-B, could also be detected on a more highly resolving SDS/PAGE gel (results not shown). Together, these data suggested that the preparation was about 50% pure. Attempts to purify this protein to homogeneity by column chromatography resulted in a loss of activity (results not shown). Activity could not be restored by reconstitution with exogenously supplied iron (dialysis overnight against 25 mM Bis-Tris buffer, pH 6.7, containing 0.1 mM FeSO₄), which suggested that the enzyme was not losing iron. The preparation of 50% purity had a peak specific activity of 32.5 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹, or 65 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ for the purified enzyme (Table 1).

Potato 13/9-LOX was very different from pea 9/13-LOX [27] in a number of biochemical properties. It exhibited considerably less interfacial reactivity with linoleate (Figure 3) [27], which

Figure 4 Effect of pH on linoleate hydroperoxidation by wild-type potato 13/9-LOX

The rates of hydroperoxidation at 234 nm for reactions of wild-type potato 13/9-LOX with linoleate in a variety of buffers of different pK_a values [27] were determined.

Table 2 Substrate and product specificity of wild-type potato 13/9-LOX

All activities were determined in 50 mM Mes, pH 5.5, at 25 °C with 0.3 mM substrate and 0.01% (v/v) Tween 20 by increase in A_{234} [hydroperoxide, HPODE + hydroxyacid, HODE (hydroxyoctadecadienoic acid) production] [27,35].

Substrate specificity	Relative rate of hydroperoxidation (% linoleate)
Linolenate	15
Arachidonate	7
Methyl linoleate	1
Product specificity	
Ratio of positional isomers of hydroperoxides (13- <i>Z</i> , <i>E</i> -HPODE/9- <i>E</i> , <i>Z</i> -HPODE) from linoleate	0.8 ($\approx$ 1:1)

suggested that potato 13/9-LOX was, like pea 13/9-LOX [27], far more effective at using the monomeric form of linoleate in the aqueous phase that was not in dynamic equilibrium with micelles. The theoretical $V_{\max}$ values for both enzymes (Table 1) were almost identical ($0.3\text{--}0.4\ \mu\text{mol}\cdot\text{min}^{-1}$ at optimum pH) but this could be approached experimentally only for potato 13/9-LOX, not for pea 9/13-LOX. This was probably due to the large difference in the K_m of linoleate (Table 1) under the assay conditions ($6\text{--}7\text{ mM}$ compared with $0.1\text{--}0.2\text{ mM}$), which indicated that the affinity for linoleate of potato 13/9-LOX was considerably higher than pea 9/13-LOX and resembled more closely that of pea 13/9-LOX [27]. Moreover, the catalytic efficiency of potato 13/9-LOX for linoleate hydroperoxidation was significantly higher than that of pea 9/13-LOX (Table 1); indeed, it was the highest of any LOX produced in this laboratory. The pH profile of linoleate hydroperoxidation for potato 13/9-LOX (Figure 4) was considerably sharper than that for pea 9/13-LOX, resembling that of pea 13/9-LOX [27], and the optimum pH was lower (5.5 for potato 13/9-LOX; approx. 6.3 for pea 9/13-LOX and pea 13/9-LOX). Potato 13/9-LOX had slightly broader substrate specificity (Table 2) than pea 9/13-LOX but not as broad as that of pea 13/9-LOX [27] and, similarly, exhibited carbonyl production from linoleate, linolenate and arachidonate (Figure 5). More interestingly, it produced roughly equimolar amounts of 9- and 13-HPODE from linoleate (or a

Figure 5 Spectral changes during reaction of wild-type potato 13/9-LOX with different fatty acids

Reactions (1 ml) of wild-type potato 13/9-LOX with 0.3 mM linoleate (top panel), linolenate (bottom panel) or arachidonate (bottom panel) in 50 mM Mes, pH 5.5, containing 0.01% (w/v) Tween 20 were scanned at 50 s intervals from 200–350 nm at 25 °C in a Shimadzu 2401-PC UV/VIS spectrophotometer.

slight molar excess of 9-HPODE; Figures 6A–6D). This was observed in crude *E. coli* extracts and for partially purified preparations. No differences were detected in the amounts of 9- and 13-HPODE that were recovered after incubation with and without extracts of *E. coli* transformed with pSBETa only. Ratios (13-HPODE/9-HPODE) of 1.09 ± 0.11 and 1.06 ± 0.13 in the absence and presence of pSBETa respectively were determined. This confirmed that impurities in the crude extracts did not invalidate their use for determining LOX positional specificity.

All potato tuber LOXs characterized to date can be described as 9-LOXs [12,13,38]. In order to justify a comparison of our product-specificity data with published data, we determined the positional specificity with linoleate for an authentic sample of a different recombinant potato tuber LOX and a crude extract from potato tubers. The major product from each reaction was 9-HPODE, consistent with observations in the literature, although 13-HPODE was produced in greater amounts from the purified recombinant LOX than would be acceptable under the definition of a 9-LOX [4], which suggests that this enzyme should be redefined as potato 9/13-LOX (Table 3). The crude potato tuber extract exhibited what can be defined as 9-LOX activity and produced a 10-fold molar excess of 9- over 13-HPODE. This confirmed that differences detected in positional specificities were not due to differences in assay procedures.

Figure 6 Product specificity of wild-type potato 13/9-LOX

Typical HPLC chromatograms of reaction mixtures: (A) auto-oxidized linoleate (40–50 °C for 24 h) to demonstrate separation of reaction products [1, 13-*Z,E*-hydroxyoctadecadienoic acid (HODE); 2, 13-*Z,E*-HPODE; 3, 13-*E,E*-HPODE; 4, 9-*E,Z*-HPODE; 5, 13-*E,E*-HODE; 6, 9-*E,E*-HPODE; 7, 9-*E,Z*-HODE; 8, 9-*E,E*-HODE]; (B) crude LOX extract from potato tubers and linoleate; (C) crude extract of *E. coli* expressing wild-type potato 13/9-LOX and linoleate; (D) purified wild-type potato 13/9-LOX and linoleate; (E) purified wild-type potato 13/9-LOX and arachidonate. Dotted arrow (B–D) refers to detection of 13-*E,E*-HODE as a shoulder in the 9-*E,Z*-HPODE peak. Products were identified from previous work [35]. Peak heights for reactions in the absence of LOX (B, D, E) or in the presence of crude *E. coli* extracts transformed with pSBETa (C) were calculated to determine the proportion of products not due to LOX (results not shown). Similar chromatograms to (C) were observed for reaction of mutant LOX in crude extracts with linoleate (results not shown).

Analysis of a potato tuber extract and purified wild-type potato 13/9-LOX immunoblotted from isoelectric-focusing gels (Figure 2C) suggested that potato 13/9-LOX was only a minor component of the total LOX population in tubers.

Table 3 Comparison of positional specificities determined for wild-type potato 13/9-LOX, other recombinant potato tuber LOXs and a crude extract from tubers

The positional specificity ratio (13-*Z,E*-HPODE/9-*E,Z*-HPODE) was determined with linoleate, except for potato 9-LOX, which was determined with linolenate and calculated from the literature [12]. The value for potato 9/13-LOX was determined for a sample of purified recombinant LOX obtained from Professor Channa Reddy [13].

LOX	Positional specificity
Potato 13/9-LOX	
Crude extract	1:1
Partially purified (Q-Sepharose)	1:1
Partially purified (peak specific activity)	1:1
Potato 9/13-LOX	1:8
Crude LOX extract from tubers	1:10
Potato 9-LOX (T8)	1:45

Figure 7 EPR spectroscopy of hydroperoxide-activated wild-type potato 13/9-LOX

(Bottom trace) Resting wild-type enzyme (85 μ M); (middle trace) after reaction at room temperature for about 20 s with a molar equivalent of 9-*E,Z*-HPODE; (top trace) after reaction at room temperature for 2 min with an additional molar equivalent of 9-*E,Z*-HPODE. The spectrum from the buffer control (50 mM Bis-Tris buffer, pH 6.7; results not shown) was subtracted to give the difference spectra shown (see the Experimental section for running conditions).

Arachidonate oxidation and leukotriene A_4 synthesis

Wild-type potato 13/9-LOX exhibited 5-LOX activity and produced 5-HPETE (and other positional isomers) from arachidonate (Figure 6E). However, the level of production of 5-HPETE was considerably lower than would have been predicted from comparing the yields of 9- and 13-HPODE from linoleate and from a comparison of the relative rates of linoleate and arachidonate hydroperoxidation at 234 nm. Due to the low product yields it was not possible to confirm the synthesis of leukotriene A_4 from detection of the hydrolysis products (Compounds I and II) by HPLC retention time. This was probably due to non-optimization of reaction conditions and the fact that only 15 % of the LOX-catalysed 5-HPETE is converted to leukotriene A_4 [36,39].

EPR spectroscopy

As observed for wild-type pea 9/13-LOX [27] and pea 13/9-LOX [27], wild-type potato 13/9-LOX was purified with the iron detectable in only the ferrous, Fe(II), state. This was in contrast to a native LOX purified from potato tubers [10] but typical of most native and recombinant LOX [40]. The spectrum of wild-

Figure 8 Protein blot analysis of wild-type and mutant potato 13/9-LOX expression

(A) Soluble protein extracts (5 μ g of total protein) from cultures of *E. coli* transformed with wild-type or mutant LOX expression constructs were separated on SDS/PAGE gels, blotted and probed with anti-B. The position of the 97 kDa marker and LOX are indicated. The dotted arrow refers to a non-full-length product or a proteolysed fragment. (B) Quantitative response between the amount of LOX-immunoreactive polypeptide detected in crude soluble protein extracts from *E. coli* as a function of total protein loaded on the blotted gel.

type potato 13/9-LOX developed a new peak at $g = 6$ after addition of 9-HPODE for 20 s (Figure 7). This signal was only 10–20% as intense as the signal developed for wild-type pea 9/13-LOX [27] but clearly had a shoulder on the low-field (higher g) side of the peak at $g = 6$. This shoulder was observed previously for wild-type pea 9/13-LOX [27]. The intensity of the $g = 6$ signal was slightly reduced in intensity after a further reaction with 9-HPODE for 2 min; this was in contrast to wild-type pea 9/13-LOX where the intensity of the $g = 6$ signal after 2 min was most intense (results not shown).

Expression and purification of mutant LOX

Expression levels of wild-type and mutant potato 13/9-LOX full-length immunoreactive protein, unlike mutant pea 9/13-LOX (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work), were more variable and mutants were generally expressed at lower levels than the wild type (Figure 8A). Typical levels of expression were: $\Delta 250 \rightarrow 254$ (62%), I437L (51%), V570I (76%) and V580F (55%). The relative activities determined in crude extracts for mutants with these levels of expression were 18, 48, 170 and 8% respectively. Thus in relation to the amount of wild-type immunoreactive protein, the relative activity of mutants $\Delta 250 \rightarrow 254$, I437L, V570I and V580F was 29, 94, 224 and 15% of the wild type respectively (Table 4). An approximately linear relationship existed between the amount of total protein (0–10 μ g) loaded on the blotted gel and the amount of LOX-immunoreactive polypeptide detected (Figure 8B).

Modelling of primary determinants of linoleate binding and positional specificity of potato 13/9-LOX and effects of mutations

The predicted amino acid sequence of potato 13/9-LOX differed by only 23 and 24 amino acids (excluding three single-residue gaps) from potato 9-LOX [12] and potato 9/13-LOX [13] respectively (results not shown). Only 16 of these residues were not modelled to lie in domain I of the proteins and some would therefore be predicted to have a role in determining positional specificity (numbering refers to potato 13/9-LOX sequence with equivalent amino acids in potato 9-LOX and potato 9/13-LOX shown in parentheses): E168 (K, K), Q212 (E, Q), I379 (V, I), V436 (V, L), I437 (L, L), I448 (T, I), T514 (S, A), S564 (M, S), V570 (I, I), M587 (M, L), K693 (E, E), T694 (R, T), D745 (E, E), R751 (K, K), V774 (I, V) and R807 (K, K). Primary sequence comparisons (Figure 1) and superposition of homology models of potato 13/9-LOX (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work), potato 9/13-LOX, potato 9-LOX and pea 9/13-LOX suggested that residues defining the linoleate-binding pocket were conserved completely, with only minor substitutions in potato 13/9-LOX of a valine (V773) for a leucine (wall of pocket), and a phenylalanine (F580) for a valine (base of pocket; Figure 9). Most of the 16 residues above were distant from the linoleate-binding pocket and iron atom; however, one of these residues (V570) was equivalent to a valine residue in pea 9/13-LOX that has been proposed to reside on the wall of the linoleate-binding

Table 4 Comparison of enzymic properties of wild-type and mutant potato 13/9-LOXs

Data (means of 4–10 replicates) were determined using linoleate unless stated. Relative activities were calculated for the same level of expression of mutant LOX as the wild type and are given as hydroperoxidation rate. Errors for K_m values are $\pm$ S.D. expressed as percentages of the mean. Positional specificity is expressed as mean $\pm$ S.D.

Property	Mutant potato 13/9-LOX				
	Wild type	$\Delta 250 \rightarrow 254$	I437L	V570I	V580F
Relative activity (% wild-type)	100	29	94	224	15
K_m (% wild-type)	100 $\pm$ 4	486 $\pm$ 64	114 $\pm$ 9	61 $\pm$ 17	384 $\pm$ 14
k_{cat}/K_m (% wild-type)	100	6	83	367	4
Substrate specificity (% linoleate)					
Linolenate	15	10	7	11	9
Arachidonate	7	4	0	0	12
Methyl linoleate	1	0	1	0	0
Positional specificity (13-Z,E-HPODE/9-E,Z-HPODE)	0.8 $\pm$ 0.2	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	0.9 $\pm$ 0.1

Figure 9 Schematic representation of linoleate binding to potato LOX

Homology models were produced for potato 13/9-LOX, potato 9-LOX, potato 13-LOX1 and potato 13-LOX2 and superimposed on a model for an energy-minimized complex of linoleate and pea 9/13-LOX (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work) to compare the residues in the putative linoleate-binding pockets. For equivalent conserved residues in potato 9/13-LOX, add three residues to those shown for potato 9-LOX.

pocket of pea 9/13-LOX. This residue was equivalent to an isoleucine in potato 9-LOX (I570) and potato 9/13-LOX (I573), but also in potato 13-LOX1 (I605) and potato 13-LOX2 (I622). This suggested that it was not a sole determinant of positional specificity and, in support of this, the effect of the mutation V570I in potato 13/9-LOX was not to change positional specificity. However, the mutation was not without effect and effectively doubled k_{cat} for this enzyme with linoleate as substrate, and was associated with a 40% reduction in K_m and nearly a 4-fold increase in catalytic efficiency. This enzyme also, unusually, showed activity with no substrate other than linoleate (Table 4). Although the same mutation in pea 9/13-LOX had no measurable effect on catalysis (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work), the residue V570 in potato 13/9-LOX, modelled on the wall of the linoleate-binding pocket, was clearly a sole determinant of linoleate binding if not positional specificity.

Differences between the residues at the base of the linoleate-binding pockets of potato 9-, 9/13- or 13/9-LOX and of potato 13-LOX1 or potato 13-LOX2 were also detected. These included substitution of a valine residue (V580) in potato 9-, 9/13- and 13/9-LOX for a phenylalanine in potato 13-LOX1 (F615) and potato 13-LOX2 (F632). The equivalent residue in cucumber 13/9-LOX was H597. Mutation of this residue to a valine produced a change in positional specificity, which suggested that the bulkiness of this residue determined positional specificity [21]; a comparison of existing potato LOX sequences would support this. However, it should be noted that wild-type cucumber 13/9-LOX exhibited dual positional specificity and produced a significant proportion (17%) of 9-HPODE; the mutant H597V did not, and produced mainly 9-HPODE [21]. Pea 9/13-LOX has a phenylalanine residue in this position, but also produced mainly 9-HPODE [27]; moreover, the effects of the mutation V580F in potato 13/9-LOX and F580V in pea 9/13-LOX (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work) had no effect on positional specificity, which confirmed that the bulkiness of this

residue was not a sole determinant of positional specificity for these LOX. Differences in residues at the base of the pocket (Figure 9) may combine to produce changes in protein conformation that could modify positional specificity without a requirement for an inverse substrate orientation. In support of this, the mutation V580F in potato 13/9-LOX was not without effect and greatly reduced k_{cat} to only 15% of the wild type, and was associated with a nearly 4-fold increase in K_m , almost complete loss (96%) of catalytic efficiency and a switch in substrate preference for arachidonate over linolenate (Table 4). Curiously, the reverse mutant (F580V) of pea 9/13-LOX was indistinguishable from the wild type (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work).

The mutation I437L was modelled some distance from the linoleate-binding pocket and so not surprisingly had no significant effect on catalysis.

Modelling of surface loop and effects on catalysis of its deletion

Modelling of potato 13/9-LOX revealed a small, very hydrophobic loop (five amino acids, IPLIL) on the surface of the protein that was about 20 Å from the carboxylate group of linoleate in the enzyme-substrate complex (Figure 10). The loop was not modelled to lie in the N-terminal β -barrel domain that has been shown to have a number of roles in catalysis, including the mediation of LOX-binding to liposomes and lipid bodies [42], calcium binding [43] and in controlling translocation between subcellular compartments [44]. Since this structure was (i) absent from a number of active LOXs, including potato 13-LOX1, potato 13-LOX2, pea 9/13-LOX, pea 13/9-LOX, rice 13-LOX1, soya bean 9/13-LOX, soya bean 13-LOX and lentil 13/9-LOX; (ii) present in all potato 9-LOXs and potato LOXs exhibiting dual positional specificity, but absent from potato 13-LOXs and (iii) somewhat remote from the linoleate-binding pocket, this suggested that the loop was not essential for correct protein folding but may have a role in catalysis. A similar

Figure 10 Modelling of surface hydrophobic loop in potato 13/9-LOX

Stereo diagram showing a C α trace of the pea 9/13-LOX homology model (R. K. Hughes, D. M. Lawson, A. R. Hornostaj, S. A. Fairhurst and R. Casey, unpublished work) in red with the longer hydrophobic loop (IPLIL, deleted in $\Delta 250 \rightarrow 254$ mutant) from the potato 13/9-LOX homology model shown in blue. Also depicted are the catalytic Fe (green sphere) and linoleic acid (black), which has been modelled in to the putative substrate-binding pocket. The Figure was produced using the programs O and OPLLOT [41].

structure has been described for lipases [45] and has been shown to have a role in determining interfacial reactions of the enzyme with its substrate, which is also not in true solution and exists as monomers and micelles in dynamic equilibrium. In the absence of micelles, the loop buries itself and prevents substrate access; above the critical micellar concentration (c.m.c.), the structure opens to allow substrate access. The kinetic properties of a native potato tuber LOX have been shown to be very dependent on the physicochemical properties of linoleate [46]. We had already observed differences in interfacial phenomena between pea 9/13-LOX and pea 13/9-LOX (loop absent) and potato 13/9-LOX (loop present) by exploring the relationships between LOX concentration and rates of linoleate hydroperoxidation at 234 nm in our standard activity assay [27]. This works well above the c.m.c. of linoleate ($< 20 \mu\text{M}$) and well below the c.m.c. of Tween 20 (6%, w/v) and suggests some dynamic equilibrium between detergent monomers and linoleate micelles [47]. Linoleate monomers must also be present since only they are small enough to be accommodated in the linoleate-binding pocket. Deletion of the loop structure in mutant $\Delta 250 \rightarrow 254$ produced a 71% reduction in k_{cat} , and was associated with a nearly 5-fold increase in K_m and almost complete loss (94%) of catalytic efficiency (Table 4). Misfolding of this mutant protein was unlikely since the extended loop is dispensable in a number of other plant LOXs and it was expressed at high levels in the soluble fraction. This suggested that the loop was involved in assisting the binding of linoleate; it was relatively remote from the binding pocket and iron atom, and we speculate that it may be involved in controlling access of the substrate. If the control mechanisms for potato 13/9-LOX and lipase are identical, then one might have predicted the deletion mutant to have activity below the c.m.c. of linoleate. Under these conditions, substrate access to the wild-type enzyme, but not the deletion mutant, would be prevented since the loop would be buried in only the former. However, both the wild-type

and deletion mutant had no detectable activity below the c.m.c. of linoleate, which suggested that either the assay conditions were not optimized, or the control mechanism was different from that of lipases.

Dual positional specificity and the role of potato 13/9-LOX in tubers

Detection of single LOX species in potato tubers that exhibit dual positional specificity has consequences for oxylipin metabolism in the plant. LOX determines the nature and supply of substrates available for other enzymes in the pathway and clearly extends the role considered for LOX in determining the 'oxylipin signature' [7] of potato tubers during their development. The identification of LOX, expressed in the plant with tissue-specific regulation during development or in response to abiotic and biotic elicitors of stress, and their characterization as recombinant proteins, should prove to be a useful approach to interpret the role of LOXs in plant biology.

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