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Purification and Characterization of a Lentil Seedling Lipoxygenase Expressed in *E. coli*: Implications for the Mechanism of Oxodiene Formation by Lipoxygenases

MARTIN P. HILBERS,[1]* ALESSANDRO FINAZZI-AGRÒ,[1]
GERRIT A. VELDINK,[2]† JOHANNES F. G. VLIEGENTHART[2]

[1]*Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Via di Tor Vergata 133, I-00135 Rome, Italy*; [2]*Department of Bio-organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands*

Lentil seedlings contain at least six lipoxygenase isoenzymes, which are difficult to separate by classical enzyme purification techniques. The aim of this work was to study one particular lentil seedling lipoxygenase, as previous work indicated possible interesting characteristics of this enzyme with respect to oxodiene formation. Since it proved to be difficult to obtain this enzyme in significant quantities in a pure state, we expressed it in *Escherichia coli*. Using an expression vector based on the T7 RNA polymerase promoter (pET11d) we achieved of a fully functional lentil seedling lipoxygenase in *E. coli*, which was purified to homogeneity by DEAE ion-exchange chromatography and gel-filtration. The products obtained from linoleic acid were analysed. This recombinant lipoxygenase corresponds to that found in the lower part of the epicotyl and in the hypocotyl of the lentil seedling. It produces predominantly 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic and minor amounts of 9(*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acids, as well as significant amounts of C_{18}-oxodienes with a regiospecificity different from hydroperoxide formation. The latter mixture was found to consist of equal amounts of 13-oxo-(9*Z*,11*E*)-octadecadienoic and 9-oxo-(10*E*,12*Z*)-octadecadienoic acids. It is concluded that (1) oxodienes formed by this lentil enzyme do not originate from a secondary conversion of hydroperoxides, but rather from a different lipoxygenase-catalysed reaction and (2) this lipoxygenase shows similarities to pea lipoxygenase G, with both representing a novel type of legume lipoxygenase.

Keywords: Plant lipoxygenase Heterologous expression Oxodiene Lipid hydroperoxide *Lens culinaris*

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INTRODUCTION

Lipoxygenases (EC 1.13.11.12, linoleate:oxygen oxidoreductases, LOX), enzymes that contain non-heme iron, catalyse the reaction of oxygen with unsaturated fatty-acids with a (1*Z*,4*Z*)-pentadiene moiety to conjugated (*Z*,*E*)-hydroperoxydienoic acids. In plants, several LOX isoenzymes usually are present, all with very similar properties (Siedow, 1991). So far, the differences between LOX isoenzymes have been

*Present address: SEQNET, Daresbury Laboratory, Warrington WA4 4AD, U.K.
†To whom all correspondence should be addressed.

Abbreviations: CP-HPLC, chiral phase HPLC; HOD, hydroxyoctadecadienoic acid; HPOD, hydroperoxy-octadecadienoic acid; IEF, iso-electric focusing; IPTG, isopropyl-1-thio-b-D-galactoside; LOX, lipoxygenase; OXOD, oxo-octadecadienoic acid; SP-HPLC, straight phase HPLC. Prefixes used with HPOD, HOD and OXOD: 9-*EZ*, 9-(10*E*,12*Z*); 9-*EE*, 9-(10*E*,12*E*); 13-*ZE*, 13-(9*Z*,11*E*); 13-*EE*, 13-(9*E*,11*E*).

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attributed solely to differences in primary structure (Siedow, 1991), so in order to investigate the properties of a single LOX isoenzyme a more fruitful approach might be to express the gene of an isoenzyme in an expression system with little or no endogenous LOX activity. This would also open the possibility of modifying the enzyme by site-directed mutagenesis. Soybean LOX-1 (Steczko *et al.*, 1991), rice seedling LOX L2 (Shirano and Shibata, 1990), a pathogen induced rice LOX (Peng *et al.*, 1994) and a potato LOX (Geerts *et al.*, 1994) have been successfully expressed in *Escherichia coli*. The expression of two pea LOXs in yeast (Knust and von Wettstein, 1992) was achieved for one of these.

Previously, we identified at least six different lipoxygenases in lentil seedlings, some of which showed a marked contrast between the positional specificity of lipid hydroperoxide and oxodiene formation (Hilbers *et al.*, 1995). Earlier proposals for the mechanism of oxodiene formation by LOX (Kühn *et al.*, 1991; Regdel *et al.*, 1985) predict similar specificities for both reactions. Since we were not able to purify one of these LOXs to homogeneity, but did isolate a full length cDNA clone for a lentil seedling LOX (Hilbers *et al.*, 1994), we decided to express this LOX in *E. coli* in order to obtain a tool for an in-depth study of this LOX.

MATERIALS AND METHODS

Construction of the expression vector pETLX2

Restriction enzymes and ligase were purchased from Pharmacia and used with the buffers supplied according to the instructions of the manufacturer. Agarose gel electrophoresis (Sambrook *et al.*, 1989) was performed with gels consisting of 1% agarose (Sigma, Wide range/Standard) in TBE buffer. Restriction fragments and PCR products were isolated from agarose gels by elution on DEAE paper (Sambrook *et al.*, 1989). We constructed a vector for the expression of a lentil seedling LOX in *E. coli* by inserting the cDNA coding for this LOX (Hilbers *et al.*, 1994) into the expression vector pET11d (Studier *et al.*, 1990), which contains the strong T7/lac promoter and the T7 terminator. The start codon for protein expression in this vector is part of a NcoI site. We changed the sequence around the LOX initiation codon to the NcoI recognition sequence (CCATGG), which does not affect the amino acid sequence of this LOX. In this way the recombinant-LOX synthesis would start exactly at the LOX initiation codon, avoiding the addition of amino acid residues to the N-terminus, which occurs with other vectors used for the expression of plant LOX in *E. coli* (Shirano and Shibata, 1990; Peng *et al.*, 1994; Geerts *et al.*, 1994). (We did not attempt to use these latter expression systems.)

A PCR-primer was designed to introduce this NcoI site at the start codon of the LOX cDNA (GAATTCCCATGGCTTCATTATTG) and a primer complementary to the 3′-end of the lentil LOX cDNA (GCGCTCTAGAATTCTTAGATGGAGATACTGTTT). With these primers (purchased from Pharmacia) and plasmid pBSLX18, which carries the entire lentil LOX cDNA, as template, a PCR product containing the entire LOX coding region was generated. PCR reaction conditions were: 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2 μM of each primer used, 200 μM of each dNTP, 20 ng template DNA and 2.5 units Taq DNA polymerase (Promega). Total volume 50 μL. The temperature program (using a Perkin Elmer Cetus DNA Thermal Cycler) was: 10 min at 94°C, followed by 30 cycles of amplification: 94°C for 30 sec, 55°C for 30 sec, and 70°C for 60 sec. The PCR product was purified by agarose gel electrophoresis, digested with NcoI and BamHI at the introduced NcoI site and the internal BamHI (1860) site of the lentil LOX cDNA, and subsequently introduced between the NcoI and BamHI sites of expression vector pET11d (Studier *et al.*, 1990), resulting in plasmid pETLX1. The possibility of frameshift errors around the NcoI site of pETLX1 was excluded by sequencing with a primer complementary to nucleotides 126–150 of the lentil LOX cDNA (Hilbers *et al.*, 1994). The remaining 910 nucleotides downstream from the internal BamHI site were taken from plasmid pBSLX1 (which is pBS containing a 3″ 2382 bp part of the lentil LOX cDNA together with a downstream BamHI site (Hilbers *et al.*, 1994)) and cloned into the BamHI site of pETLX1, resulting in the LOX expression vector pETLX2. Orientation of the inserted BamHI fragment in the expression vector was confirmed by restriction analysis. The entire construction procedure was performed twice, starting from two different PCR reactions.

Expression of lentil LOX in E. coli

The pETLX2 expression vector was used to transform *E. coli* strain BL21(DE3), using the

procedure of Hanahan (1983). Transformation of this *E. coli* strain proved to be about 500 times less efficient than the transformation of K strain *E. coli*. BL21(DE3) carries the gene for T7 RNA polymerase, controlled by the lacUV promoter. The transformed bacteria were grown at 37°C in 50 ml LB medium containing 80 μg/ml ampicillin until a cell density of $A_{550} = 0.5$ was reached. This usually took 2.5 hr, starting with an inoculation from a fresh overnight culture of LOX-expressing bacteria. LOX expression then was induced by adding isopropyl-1-thio-b-D-galactoside (IPTG) to a final concentration of 0.1 mM, and the cells were grown further for 16 hr at 15°C. Cells were harvested by centrifugation (6000 ***g***, 10 min, 4°C), briefly washed with water, resuspended in 5 (v/w) vols of extraction buffer (30 mM sodium phosphate, 10 mM EDTA, pH 7.0) and disrupted by sonication. The soluble material (supernatant) was collected following centrifugation (15,000 ***g***, 30 min, 4°C) and assayed for LOX activity.

Purification

All manipulations after harvesting the bacteria were performed at 4°C. A crude extract was prepared from 1 l of LB medium inoculated with a fresh overnight culture of the bacteria carrying the expression vector. The supernatant of the centrifugation was dialysed twice, using standard dialysis tubing with a nominal MW-cutoff of 12,000, against 1 l of column buffer (0.01 M sodium phosphate, 1 mM EDTA, pH 7.2) and loaded on a DEAE–Sepharose column (200 × 20 mm) equilibrated with column buffer. LOX was eluted with a linear gradient of 0–1 M NaCl in 400 ml of column buffer (45 ml/hr). LOX-containing fractions were pooled and concentrated in an ultrafiltration unit (Amicon YM-30 membrane). The concentrate was then further purified by Sephadex G150-Superfine gel-filtration column (600 × 25 mm) chromatography. LOX-containing fractions were pooled and concentrated as above. The LOX preparation was then brought to 20% (v/v) glycerol and stored at −80°C until further analysis.

Lipoxygenase assay and incubations

LOX activities in bacterial extracts and column fractions were measured spectroscopically (Axelrod *et al.*, 1981) at room temperature in a 1 ml cuvette (optical pathway 1 cm) by adding a quantity of the LOX-containing solution, adjusted to give an increase of 0.02–0.2 absorbance units per min, to an 80 μM solution of linoleic acid in 0.1 M sodium phosphate, pH 7.0, and recording the increase in absorbance at 234 nm. A single unit of activity was defined as the amount of enzyme catalysing the synthesis of 1 μmol of hydroperoxides per minute. LOX activity towards α-linolenic acid was measured in the same manner with 80 μM α-linolenic acid. Oxodiene formation was measured by recording the absorbance at 285 nm. The pH-optimum was determined by measuring LOX activity with 0.1 M sodium acetate (pH 3–5.5), 0.1 M sodium phosphate (pH 5.5–8.0) and 0.1 M sodium borate (pH 8.0–10.0) buffers. For the overlapping pH values, measurements in both buffers were performed. UV absorbance measurements were performed on a Uvikon 860 spectrophotometer or on a Hewlett Packard HP8452A diode array spectrophotometer. Protein concentrations were determined using the bicinchoninic acid method (Smith *et al.*, 1985) with bovine serum albumin as standard.

For product analysis 25 ml of a 0.1 mM solution of linoleic acid in 0.1 M sodium phosphate, pH 6.5, was incubated with 0.1 unit of the purified lipoxygenase. The reaction was allowed to proceed for 3 hr at 0°C in a 100% oxygen atmosphere, leading to 78% conversion of the linoleic acid. The reaction was then stopped by acidification to pH 3.0, and the products were extracted with octadecyl solid-phase extraction columns (J. T. Baker). The products were converted into methyl esters with ethereal diazomethane, dissolved in hexane and analysed by HPLC within 48 hr.

Electrophoresis and immunoblotting

SDS–PAGE (Laemmli, 1970) was performed on a SE-250 Mighty Small II vertical electrophoresis apparatus (Hoefer). Non-denaturing isoelectric focusing (IEF), with Ampholine pH 5–7, (Sigma) was performed on the same apparatus using the procedure of Robertson *et al.* (1987) with 25 mM phosphoric acid as anode solution and 25 mM NaOH as cathode solution. Samples were loaded on the acidic part of the gel and IEF was performed for 1.5 hr at 200 V followed by 1 hr at 300 V. SDS–PAGE and IEF gels were stained with Coomassie Brilliant Blue R250 or were blotted on Hybond-N membranes (Amersham) with a Mini Protean protein transfer cell (Bio-Rad). LOX was immunodetected with mouse monoclonal

antibodies against soybean LOX-1 (Maccarrone *et al.*, 1992). Alkaline phosphatase conjugated goat anti mouse IgG (Sigma) was used as secondary antibody and the blot was stained with Sigma Fast BCIP/NBT. Molecular masses and isoelectric points were estimated from the position of marker-proteins on a separate part of the gel, stained with Coomassie Brilliant Blue R250. Molecular mass markers (Sigma) were chicken muscle pyruvate kinase (58 kDa), rabbit muscle fructose-6-phosphate kinase b (84 kDa), *E. coli* β-galactosidase (116 kDa) and human plasma α_2-macroglobulin (180 kDa). pI markers (Sigma) were bovine erythrocyte carbonic anhydrase II (pI 5.4 and 5.9) and human erythrocyte carbonic anhydrase II (pI 6.6).

Analytical

Methyl esters of the reaction products were analysed by straight phase HPLC (SP-HPLC) on a CP-Sphere Si column (Chrompack, 5 μm particles, 4.6 × 250 mm) using an HP1040A diode array UV detector (Hewlett Packard) and HP7994A analytical workstation (Hewlett Packard) for data processing. Spectra of peaks were recorded at 2 nm resolution. Products were eluted isocratically with *n*-hexane/diethyl-ether/HAc (90:10:0.1, v/v) (Teng and Smith, 1985) at a flow of 3.0 ml/min, which allowed a complete separation of oxodiene, hydroperoxide and hydroxide products. Relative amounts of the different products were calculated from the peak areas, assuming a molar absorbance of 22,000 at 268 nm for oxo-octadecadienoic acids (OXODs) and 25,000 at 234 nm for hydroperoxy-octadecadienoic acids (HPODs) and hydroxy-octadecadienoic acids (HODs). Esterified HPODs were collected, reduced with a 10-fold molar excess of $NaBH_4$ (in methanol, 20 min, room temperature) and the resulting 13-*ZE*-HOD and 9-*EZ*-HOD methyl esters were isolated for chiral analysis using the same SP-HPLC system. The 13-*ZE*-HOD and 9-*EZ*-HOD methyl ester peaks from the LOX reaction were also collected for chiral analysis.

The absolute configuration of the products was determined by chiral phase HPLC (CP-HPLC) on a Chiralcel OD-R column (J. T. Baker). Peaks were identified by comparison with authentic 13*S*-*ZE*-HPOD and 9*S*-*ZE*-HPOD, prepared from linoleic acid with soybean LOX-1 (Gardner, 1989b). Products were eluted with MeOH/H_2O/HAc (85:15:0.1 v/v).

Mass spectra were obtained on an MD800/8060 GC-MS system (Fisons instruments; electron energy 70 eV) equipped with a DB-1 fused-silica capillary column (30 m × 0.32 mm, J and W Scientific). Methyl esters of OXODs were analysed without further derivatization (time program: 2 min at 180°C, then increasing with 5°C/min to 250°C. Injection temperature 250°C, ion source temperature 200°C). Esterified HPODs were reduced to HOD methyl esters with a 10-fold molar excess of $NaBH_4$ (in methanol, 20 min, room temperature). The corresponding hydroxystearates were obtained by bubbling hydrogen gas through a methanolic solution of the product in the presence of PtO_2. Hydroxy-stearates and hydroxylinoleates were converted into trimethylsilylethers by dissolving them in pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane (5:2:1, v/v). The silylated products were then analysed by GC–MS (see above, time program: 2 min at 140°C, then with 4°C/min to 280°C)

RESULTS

Expression of lentil seedling LOX in E. coli

Active LOX was obtained from the BL21(DE3)/pETLX2 expression system by lowering the growth temperature below 30°C after induction with IPTG. UV-spectra of an incubation of linoleic acid with bacterial extracts showed the formation of a product with an absorbance maximum at 234 nm. No such product was obtained with an extract of bacteria carrying the pET11d vector without the LOX insert. SDS–PAGE analysis of the bacterial extract showed a new band with a molecular mass of about 94 kDa, which was recognized by antibodies against soybean LOX-1 (Fig. 1). Optimal conditions for LOX expression were induction with 0.1 mM IPTG followed by growth at 15°C. Higher IPTG concentrations resulted in less active extracts and slowed down bacterial growth. The BL21(DE3)/pETLX2 expression system we constructed starting from a different initial PCR reaction resulted in a system with nearly identical LOX activity, so it is unlikely that any mutation had occurred during the PCR step.

In preliminary experiments (data not shown) we also used an expression vector (pBTac1, Boehringer-Mannheim) with the LOX cDNA under control of the tac promoter. With a K strain host like JM109 no active LOX could be obtained under conditions that gave active LOX with the system described above, and all material cross reacting with LOX antibodies

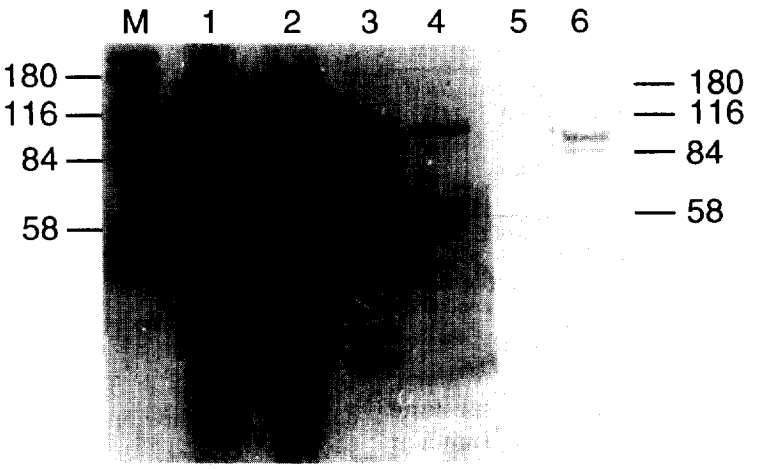

Fig. 1. SDS–PAGE analysis. Electrophoresis was performed under reducing conditions on a 10% polyacrylamide gel. Lanes 1–4: Coomassie stained gel; lanes 5–6: Western blot of a gel loaded with the same samples as lanes 1 and 2, immunodetected with antibodies to soybean LOX-1. M: markers. Lane 1: extract from BL21(DE3) carrying pET11d (100 mg). Lane 2: extract from BL21(DE3) carrying pETLX2 (100 mg). Lane 3: partial purified LOX after DEAE–Sepharose chromatography (20 μg). Lane 4: purified LOX after gel-filtration (5 μg).

was found in the insoluble fraction of bacterial extracts. With strain BL21(DE3) and the pBTac1–LOX expression vector, active LOX was obtained, although only 20% of the yield obtained with the pETLX2 vector.

Since LOX requires iron as a cofactor we also investigated the influence of iron addition to the medium. *Escherichia coli* has an uptake system for Fe(II) (Kammler *et al.*, 1993), but the addition of neither $FeSO_4$ nor FeH_2(EDTA) to the medium, in the range of 0.5–500 μM, had any influence on LOX activity.

Purification and characterization

Chromatography of an extract from bacteria expressing lentil LOX on a DEAE–Sepharose column (Fig. 2) resulted in elimination of most of the non-LOX proteins. The major contaminant remaining was a 30 kDa protein, which was absent in extracts of bacteria without the LOX–cDNA in the expression vector. This 30 kDa band was recognized by antibodies to LOX, therefore it was probably a partially synthesized LOX peptide, or a degradation product. Gel filtration (Fig. 2) of the LOX fraction from the DEAE column afforded separation of the active LOX from the remaining contaminants (Table 1). SDS–PAGE (Fig. 1) showed the LOX to be pure after this step.

Previously, we used western blots of IEF gels to identify four different LOXs, i.e. lentil LOX E1, E2, H1 and H2, in the vegetative tissues of lentil seedlings (Hilbers *et al.*, 1995). A comparison of the isoelectric point of the lentil LOX obtained from *E. coli* with those of these four LOXs (Fig. 3) shows that this LOX is probably identical to LOX H2, which is found in the lower part of the epicotyl and in the hypocotyl of the seedling.

For the recombinant lentil LOX we determined the reactivity towards linoleic acid and

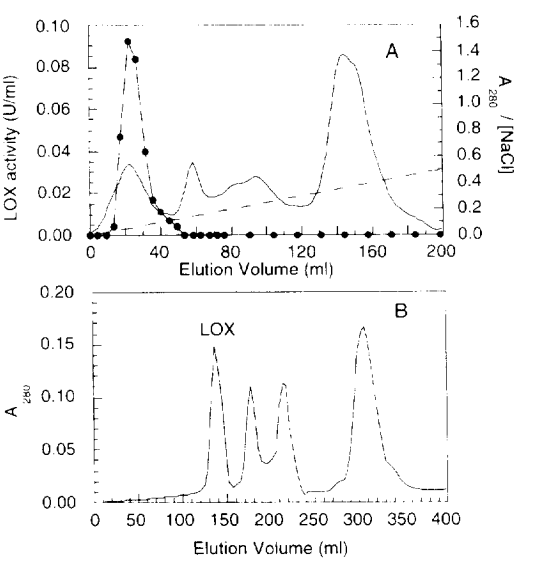

Fig. 2. Purification of the lentil seedling lipoxygenase expressed in *E. coli*. (A) DEAE–Sepharose chromatography of the crude *E. coli* extract: LOX activity shown in fractions (–•–), absorbance at 280 nm (———) and the NaCl concentration in the eluent (– – –). (B) Gel-filtration of the partially purified LOX from the DEAE–Sepharose column. Fractions were too dilute for direct LOX assays; therefore, the four peaks were pooled, concentrated and assayed for LOX activity. Only the peak with LOX activity is indicated.

Table 1. Purification of lentil seedling LOX expressed in *E. coli**

Purification step	Protein (mg)	LOX (units/mg)	Purification (fold)	Yield (%)
Crude extract	253	0.022		100
Ion exchange	14	0.35	16	88
Gel filtration	2.7	1.4	64	68

*Purification was started with 8 g (wet weight) of *E. coli* cells obtained from 1 l of LB medium. One unit of LOX is the amount of LOX catalysing the synthesis of 1 μmol of hydroperoxide per minute under the assay conditions; see Materials and Methods.

α-linolenic acid. With linoleic acid as substrate we determined the pH optimum and the amount of oxodienes formed during the reaction (Table 2). In Fig. 4 the spectral change during an incubation of linoleic acid with this LOX is shown. The increase of absorbance at 285 nm indicates that oxodienes were formed in addition to hydroperoxides. The same experiment as shown in Fig. 4 was performed with 80 μM 13-*ZE*-HPOD instead of linoleic acid to investigate whether the oxodienes result from a conversion of the primary reaction product, or from a different direct pathway catalysed by LOX. The latter possibility proved to be correct because no conversion of HPOD into OXOD was observed.

Product analysis

Methyl esters of the reaction products obtained from linoleic acid with the recombinant lentil LOX were analysed by SP-HPLC. The chromatograms (Fig. 5) showed four peaks

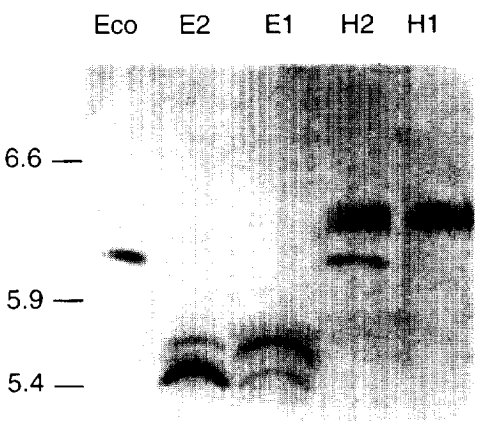

Fig. 3. Western blot of an IEF gel (pI 5–7, see text) loaded with the lentil seedling lipoxygenase expressed in *E.coli* (0.01 unit of enzyme activity) and four partially purified lentil seedling lipoxygenases (0.05 units), immunostained with antibodies against soybean LOX-1. Eco: LOX expressed in *E.coli*. E2, E1, H2, H1: lentil seedling lipoxygenases E2, E1, H2 and H1, respectively. The positions of IEF markers are indicated.

(A–D) with an absorbance maximum at about 268 nm, identified as 13-EZ-, 9-*ZE*-, 13-*EE*- and 9-*EE*-OXOD (Sanz *et al.*, 1993), respectively, followed by eight peaks (1-4, I–IV) with an absorbance maximum at about 234 nm, identified as 13-*ZE*-, 13-*EE*-, 9-*EZ*- and 9-*EE*-HPOD, followed by 13-*ZE*-, 13-*EE*-, 9-*EZ*- and 9-*EE*-HOD (Teng and Smith, 1985), respectively.

The peak assigned to 13-*ZE*-HPOD coeluted with authentic 13-*ZE*-HPOD obtained with soybean LOX-1. The 9-*EZ*-HPOD peak coeluted with the minor product obtained with soybean LOX-1 at pH 6.5, known to be 9-*EZ*-HPOD (Gardner, 1989a). *EE*-isomers of conjugated dienes have absorbance maxima at lower wavelengths than *EZ*- or *ZE*-isomers (Lezerovich, 1986), and this was also found for the peaks assigned to these isomers (for OXODs 267 versus 269 nm, for H(P)ODs 231 versus 233 nm). Reduction of an OXOD-isomer or HPOD-isomer with $NaBH_4$ yielded a product coeluting with the corresponding HOD-isomer. Mass spectra of esterified 9-*EZ*-OXOD and 9-*EE*-OXOD showed fragments at *m/z* 99 and 252, whereas 13-*ZE*-OXOD and 13-*EE*-OXOD methyl esters showed fragments at *m/z* 166 and 185, as expected for such products (Kühn *et al.*, 1991). The molecular ion for the OXOD methyl esters was found at *m/z* 308. Mass spectra of the HPOD methyl esters, after reduction, hydrogenation and silylation, and of the HOD methyl esters, after hydrogenation and silylation, confirmed the assignment of positional isomers. The 9-products all showed fragments at *m/z* 229 and 259, whereas the 13-products had fragments at *m/z* 173 and 315. Mass spectra of the non-hydrogenated compounds showed the molecular ion at *m/z* 382, as expected. The fragment at *m/z* 311 was more abundant than that at *m/z* 225 for the 13-products, whereas the reverse was found for the 9-products (Hubbard *et al.*, 1980).

CP-HPLC on a Chiralcel OD-R column afforded separation of *R* and *S* enantiomers of 13-*ZE*-HOD and 9-*EZ*-HOD methyl esters. The peaks assigned to *S*-enantiomers coeluted with the products obtained with soybean LOX-1 at pH 6.5, being *S*-enantiomers of both 13-*ZE*-HPOD and 9-*EZ*-HPOD (Gardner, 1989a).

In Table 2 the results of the various analyses are shown, together with the comparison with results we obtained with partially purified lentil LOXs (Hilbers *et al.*, 1995). The SP-HPLC

Table 2. Characteristics of the lentil LOX expressed in *E. coli* and comparison with partially purified lentil seedling LOXs. About 10% of the products formed were HODs, which showed the same isomeric and enantiomeric distribution as the HPODs. Thus, the HODs originate from the LOX reaction and in this table data for HODs and HPODs are combined

Lipoxygenase preparation	H1	H2	*E. coli*
pI	6.3	6.3 6.1	6.1
pH optimum	5.75	6.5	6.5
Reactivity to α-linolenic acid[a]	46%	17%	5%
H(P)OD/OXOD activity[b]	64:36	58:42	55:45
Product distribution[c]	84:16	88:12	87:13
Regiospecificity H(P)OD[d]	33:10:46:8	56:7:28:9	79:3:14:4
Regiospecificity OXOD[d]	40:17:33:10	39:12:41:8	39:9:44:8
Enantiospecificity 13-*ZE*-HPOD[e]	28:72	21:79	15:85
Enantiospecificity 9-*EZ*-HPOD[e]	52:48	47:53	41:59

[a]Relative reaction rate with α-linolenic instead of linoleic acid.
[b]Ratio of activities measured at 234 and 285 nm.
[c]Molar ratio of H(P)OD:OXOD formed during reaction (determined by SP-HPLC).
[d]Ratio of 13-*ZE*-:13-*EE*-:9-*EZ*-:9-*EE*- isomers (determined by SP-HPLC).
[e]Ratio of *R*:*S* enantiomers (determined by CP-HPLC)

results show a marked contrast in specificity of the recombinant lentil LOX with respect to HPOD and OXOD formation. The HPOD formed is mainly 13-(*S*)-*ZE*-HPOD, whereas equal amounts of 9-*EZ*-OXOD and 13-*ZE*-OXOD are formed.

DISCUSSION

We achieved the expression of a lentil seedling LOX in *E. coli* BL21(DE3) with the pETLX2 vector. In order to obtain active plant LOX from bacteria it has so far been necessary to grow the bacteria at low temperatures (Steczko *et al.*, 1991; Shirano and Shibata, 1990; Peng *et al.*, 1994; Geerts *et al.*, 1994) and this proved also to be the case for the lentil LOX. We could not obtain active LOX with a tac-promoter-based expression vector in a K12-strain host, but succeeded when using this vector with BL(21)DE3. The B-strain BL21(DE3) lacks the lon and ompT proteases and this is apparently of importance for successful expression of LOX in *E. coli*.

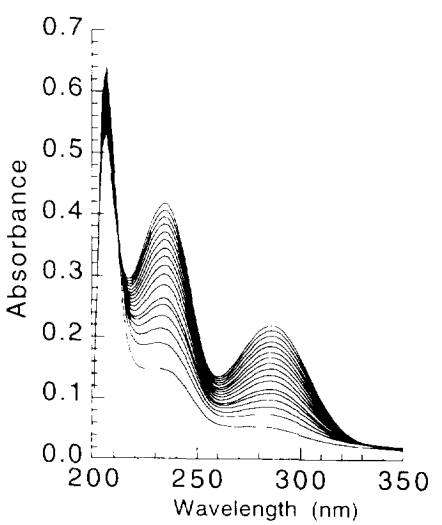

Fig. 4. Spectral changes during an incubation of linoleic acid with the lentil LOX purified from *E. coli*. The reaction was started by adding linoleic acid from a 10 mM stock solution (Axelrod *et al.*, 1981) to 1 ml of an air-saturated 0.1 M sodium phosphate buffer (pH 6.8) containing 5 mU of the purified lipoxygenase. The final linoleic acid concentration was 80 μM. Spectra were recorded with 15 s intervals in a HP8452A diode array spectrophotometer with a resolution of 2 nm.

The characteristics of the LOX obtained from *E. coli* are similar to those of the partially purified lentil LOX-H2, and most differences can be attributed to the contamination of LOX-H2 with lentil LOX-H1. The activity of the recombinant LOX is lower than that of lentil LOX H2. Since we performed the vector construction in duplicate, resulting in equally efficient expression systems, it is unlikely that this is a result of mutations during the PCR reaction. It is more likely that it is a result of misfolding in the bacteria. As the characteristics of the recombinant LOX are as expected for pure lentil LOX H2, we conclude that the recombinant LOX consists of a fully active and a completely inactive fraction, resulting in apparent low overall activity. The system is therefore suitable to study specificity of the enzyme.

The purified recombinant lentil LOX has several interesting characteristics. It produces 13-(*S*)-*ZE*-HPOD, but also oxodienes, which are, however, synthesized in a non-regiospecific manner, comprising equal amounts of 13-*ZE*-OXOD and 9-*EZ*-OXOD. Most LOXs that

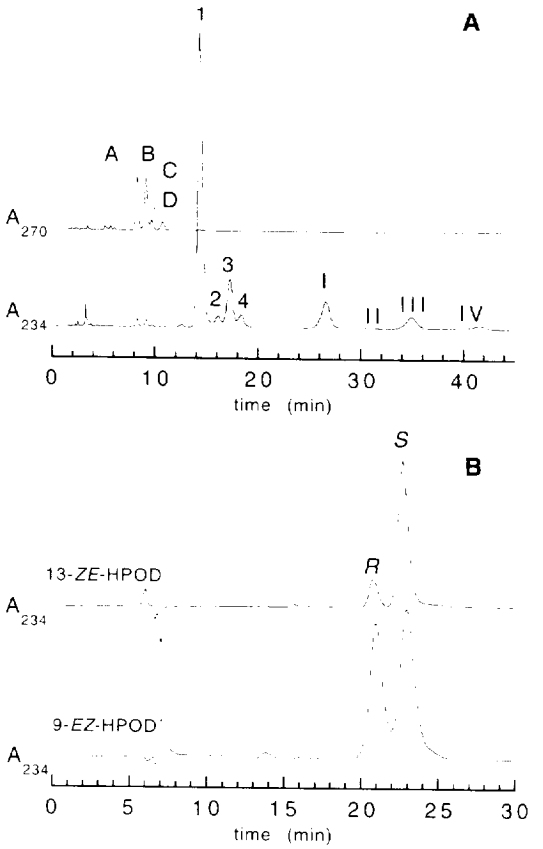

Fig. 5. HPLC analysis of the products obtained with the lentil LOX expressed in *E. coli*. (A) SP-HPLC chromatogram of the esterified reaction products. Time-traces recorded at 270 and 234 nm are shown on the same scale. Peak identification (see text) (A) 13-*ZE*-OXOD; (B) 9-*EZ*-OXOD; (C) 13-*EE*-OXOD; (D) 9-*EE*-OXOD; (1) 13-*ZE*-HPOD; (2) 13-*EE*-HPOD; (3) 9-*EZ*-HPOD; (4) 9-*EE*-HPOD; (I) 13-*ZE*-HOD; (II) 13-*EE*-HOD; (III) 9-*EZ*-HOD; (IV) 9-*EE*-HOD. (B) CP-HPLC analysis. The esterified hydroperoxides were reduced to hydroxy compounds for CP-HPLC. Positions of the *R* and *S* enantiomers are indicated.

synthesize oxodienes are not very specific in their positional specificity of HPOD synthesis, and have a preference for making a slight excess of 9-*EZ*-HPOD (Kühn *et al.*, 1991; Siedow, 1991; Sanz *et al.*, 1993). It has been proposed that OXODs originate from a conversion of HPODs (Kühn *et al.*, 1991), but this is difficult to reconcile with the difference in specificity observed between OXOD and HPOD formation with the lentil LOX. Furthermore, no conversion of HPOD into OXOD by the lentil LOX could be demonstrated, and also oxodiene forming LOXs from kidney bean and pea do not convert HPOD into OXOD (Sanz *et al.*, 1993). The alkoxy-radical that results from activation of the enzyme by 13-*EZ*-HPOD (Fig. 6) has been proposed to be a source of oxodienes (Regdel *et al.*, 1985). However, this should result in the formation of 13-*EZ*-OXOD, which conflicts with our results. Oxodiene formation is inhibited by antioxidants, whereas the HPOD formation is relatively insensitive to antioxidants (Maccarrone *et al.*, 1995; Regdel *et al.*, 1985).

The LOX reaction cycle (Fig. 6) (De Groot *et al.*, 1975) is thought to start with the abstraction of a hydrogen from the central methylene group of the substrate, leading to a fatty acid radical bound to Fe(II) LOX. Instead of proceeding along the normal enzymatic cycle, it is possible that the radical dissociates from the enzyme and reacts in an aspecific manner with molecular oxygen to a peroxy-radical. The lack of specificity of the OXOD forming reaction would be explained by the conversion of this radical into OXOD. However, the manner in which this conversion may happen remains to be elucidated (Gardner, 1989b). One might speculate that LOX is involved in this step, through the oxidation of the Fe(II)-LOX to the active Fe(III)-state by the peroxy-radical, resulting in OXOD and OH^- (Fig. 6). Also, the activation of Fe(II)-LOX by HPOD yields OH^- (De Groot *et al.*, 1975). Interestingly, recent X-ray absorption (Scarrow *et al.*, 1994) and near infra-red magnetic circular dichroism (Pavlosky and Solomon, 1994) experiments indicate the presence of a hydroxy ligand at iron in Fe(III)-LOX, which is absent in the inactive

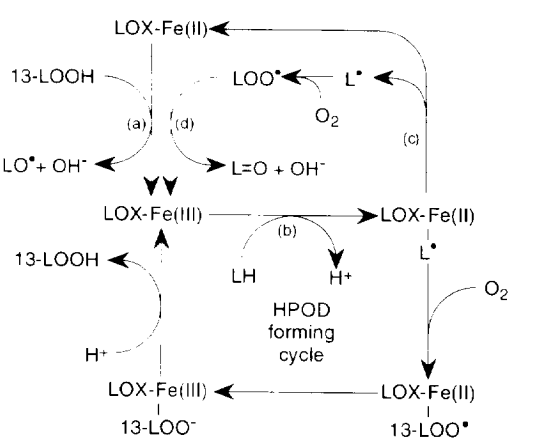

Fig. 6. Lipoxygenase catalytic cycle: (a) activation of the enzyme by hydroperoxide product (LOOH); (b) hydrogen abstraction from linoleic acid (LH) resulting in a fatty acid radical (L•). The normal lipoxygenase reaction then proceeds following the HPOD forming cycle. OXOD formation may start with release of the fatty acid radical (c), which then reacts in an aspecific manner with molecular oxygen to a peroxy radical. Oxodienes (L=O) might be formed from the peroxy radicals by hypothetical step (d) (dashed arrows).

Fe(II) enzyme. The activation by a fatty acid peroxy radical would explain why LOX remains active despite the inactivation that should result from the dissociation of the fatty acid radical. Furthermore, it explains the lack of radical recombination products in reactions with OXOD-forming LOXs (Kühn *et al.*, 1991; Regdel *et al.*, 1985).

The low reactivity towards α-linolenic acid is also remarkable. For six soybean LOXs the reaction rate with α-linolenic was between 88 and 447% of that with linoleic acid (Kato *et al.*, 1992a) and rates with LOXs from other plants usually fall within this range. It is unlikely that this lentil LOX is involved in the biosynthesis of jasmonic acid, a function frequently described as being one of the physiological functions of LOX (Siedow, 1991; Vick and Zimmerman, 1987), because it requires the conversion of α-linolenic acid by LOX.

Since we published the primary structure of this lentil LOX (Hilbers *et al.*, 1994), a few new legume LOX sequences have been published. One of them, pea LOX-G (Rodriguez-Concepcion and Beltran, 1995) has 82.4% amino-acid residues identical to the lentil LOX, whereas other legume LOXs have a sequence-homology to that of lentil LOX of around 70%. Furthermore, both LOXs are abundant in seedlings, therefore it is likely that this pea LOX, which is involved in carpel development, is the pea counterpart of lentil LOX-H2.

We found LOX-H2 to be present in the lower part of the epicotyl and in the hypocotyl of the lentil seedling (Hilbers *et al.*, 1995). This enzyme is a minor LOX in the shoot-tips and absent in the root. Park *et al.* (1994) have used two cDNA probes to investigate the localization of LOX transcripts in soybean seedlings. One of their probes (pTK18) corresponds to soybean LOX-4 (Kato *et al.*, 1993), which has been identified as a vegetative storage protein (Tranbarger *et al.*, 1991). This probe hybridizes with RNA from all the vegetative parts of the soybean seedling, indicating that soybean LOX-4 is to be found in all tissues. Soybean LOX-4 produces a slight excess of 9-*EZ*-HPOD over 13-*ZE*-HPOD, the reaction rate with α-linolenic acid is 293% of that with linoleic acid (Kato *et al.*, 1992a) and its amino-acid sequence (Kato *et al.*, 1993) has only 70% homology towards the lentil LOX sequence. The other probe of Park *et al.* (1994), pTK11, corresponds to soybean LOX-6 (Kato *et al.*, 1992b; Shibata *et al.*, 1991) and hybridizes predominantly with RNA from the lower part of the hypocotyl/radicle. It produces mainly 13-*ZE*-HPOD, as does lentil LOX-H2, but it is far more reactive (311% of the reaction rate with linoleic acid) with α-linolenic acid (Kato *et al.*, 1992a). The homology of the soybean LOX-6 sequence with that of lentil LOX is only 66%. Thus, these two vegetative soybean LOXs differ too much in distribution pattern, enzymatic properties and amino-acid sequences from lentil LOX H2 to be a soybean counterpart of this lentil LOX. The morphology of soybean and lentil seedlings is rather different, with the lentil epicotyl already starting to grow within 2 days of the onset of germination, without the development of primary leaves from the cotyledons. With soybean the growth of the epicotyl starts much later, after the development of primary leaves from the cotyledons. If a soybean counterpart of the lentil LOX-H2 exists, it might appear later in the development of the soybean seedling, when the epicotyl starts to grow.

We conclude that the LOX we expressed in *E. coli*, and its pea counterpart, pea LOX-G (Rodriguez-Concepcion and Beltran, 1995), are novel types of LOX, different from other described LOXs. Moreover, the results with this LOX offer new insights into the mechanism of oxodiene formation by LOXs.

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