ELSEVIER

Plant Science 111 (1995) 169–180

PLANT SCIENCE

Heterogeneity and developmental changes of lipoxygenase in etiolated lentil seedlings

Martin P. Hilbers[a,1] Bas Kerkhoff[b], Alessandro Finazzi-Agrò[a], Gerrit A. Veldink*[b], Johannes F.G. Vliegenthart[b]

[a]*Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Via di Tor Vergata 135, I-00133 Rome, Italy*

[b]*Department of Bio-organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands*

Received 3 July 1995; revision received 4 September 1995; accepted 8 September 1995

Abstract

Lipoxygenase activity in developing etiolated lentil seedlings showed a continuous decline in the cotyledons, whereas the lipoxygenase activity in the expanding tissues increased up to 6 days after germination, followed by a decrease. SDS-PAGE and IEF analysis showed the presence of at least two different lipoxygenases in the cotyledons and of four distributed over the vegetative parts. Characterization of these six lipoxygenases after partial purification showed that they differed in p*I*, molecular mass, substrate preference and product specificity. The products obtained by incubating linoleic acid with these lipoxygenases were analysed by straight phase HPLC and chiral phase HPLC. One of the seed lipoxygenases and two shoot-tip specific lipoxygenases synthesized mainly 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid. The other seed lipoxygenase and a root specific lipoxygenase synthesized 9-(*R*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid and 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid in a molar ratio of 2:1 and produced also oxodienes. A lipoxygenase found in the lower part of the epicotyl and in the hypocotyl synthesized 13-(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid and oxodienes.

Keywords: *Lens culinaris*; Lipoxygenase; Germination; HPLC; Isoenzymes; Oxodiene

Abbreviations: CD, circular dichroism; CP-HPLC, chiral phase-high performance liquid chromatography; HOD, hydroxy-octadecadienoic acid; HPOD, hydroperoxy-octadecadienoic acid; IEF, iso-electric focusing; LOX, lipoxygenase; OXOD, oxo-octadecadienoic acid; Prefixes used with HOD, HPOD 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*); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SP-HPLC, straight phase-high performance liquid chromatography.

* Corresponding author, Fax: 31 30 540980; E-mail: veldink@cc.ruu.nl.

[1] Present address: SEQNET, Daresbury Laboratory, Warrington WA4 4AD, England, UK.

1. Introduction

Lipoxygenases (EC 1.13.11.12, linoleate:oxygen oxidoreductase, LOX) catalyse the dioxygenation of fatty acids containing a (1*Z*,4*Z*)-pentadiene moiety. These enzymes are ubiquitous in plants [1], have been found in mammals [2] and have further been reported to occur in yeast [3], algae [4] and bacteria [5].

In higher plants, the seeds of legumes are par-

0168-9452/95/$09.50
SSDI 0168-9452(95)04240-U

ticularly rich sources and, since LOX-1 from soybean is abundant and easy to purify, most data concerning structure and mechanism of LOX originates from experiments with this enzyme [1]. The occurrence of lipoxygenases is not restricted to seeds. For many plants, germination is accompanied by high LOX activities in the newly developing tissues [6–9]. The enzymatic activities decrease as the tissues mature, but LOX can still be found in mature tissues such as soybean leaves [10], tomato fruits [11] or potato tubers [12]. Usually, several isoenzymes are found which differ in various properties such as pH-optimum, substrate preference, product specificity and p*I*.

Although many research efforts have been directed at the structure and mechanism of LOX, little is known about its physiological function in plants. The high LOX levels often found during germination suggest a role in growth and development [13]. Increased LOX levels are connected to wounding and pest attacks, which indicates a possible function in counteracting these events [14]. LOX products are possible precursors for physiologically important compounds like jasmonic and traumatic acid [15].

The abundance of LOX in lentil seeds (*Lens culinaris* Medik.) has been recognized [16] and lentils are richer in LOX than soybean seeds, if assayed at neutral pH [17]. Eskin and Henderson [18] found three LOX isoenzymes in lentils, which they did not further characterize.

Most studies focus on a single LOX-isoenzyme, or regard different isoenzymes from the same tissue at a certain developmental stage of the plant. The distribution of different isoenzymes in the entire plant usually receives little attention. The aim of this investigation was to analyse the LOX activity during lentil germination and to localize and characterize the different LOX-isoenzymes throughout the plantlet.

2. Materials and methods

2.1. Plant material

Red lentils (*Lens culinaris* Medik. ssp *microsperma*) were obtained from a local grocery. Lentils were sterilized by rinsing them briefly with 96% ethanol. The seeds were soaked for 24 h in autoclaved tap water. The moment of imbibition was taken as the starting point of germination. The seedlings were allowed to grow on moist sawdust in the dark at 25°C. They were watered daily with autoclaved tap water. Harvested seedling parts were homogenized with 3 ml assay buffer (0.1 M sodium phosphate, 1 mM EDTA, 0.1% Triton X-100, pH 6.8) per g of tissue, using an Ultra Turrax T25 homogenizer. Homogenates were centrifuged for 30 min at 20 000 × *g*, the resulting supernatants were recentrifuged for 10 min at the same speed and the final supernatants were assayed immediately for LOX activity.

2.2. Lipoxygenase assay and incubations

LOX activity was measured spectroscopically [19] 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 of absorbance at 234 nm. One unit of activity was defined as the amount of enzyme catalysing the synthesis of 1 μmol of HPOD/min. LOX activity towards α-linolenic acid was measured in the same manner with 80 μM α-linolenic acid. OXOD formation was measured by recording the increase of absorbance at 285 nm. pH-optima were determined by measuring LOX activities 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-spectroscopy was performed on a Uvikon 860 spectrophotometer. Protein concentrations were determined using the bicinchoninic acid method [20] 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, or in 0.1 M sodium borate, pH 9.0, was incubated with 0.5 U of the lipoxygenase preparation to be investigated. The reaction was allowed to continue for 1.5–2.5 h at 0°C in a 100% oxygen atmosphere until at least 80% of the linoleic acid was converted to products. The reaction was then stopped by acidification to pH 3.0 and the products were extracted with octadecyl solid-phase ex-

traction columns (J.T. Baker). The products were esterified with ethereal diazomethane, dissolved in hexane and analysed by HPLC within 48 h. As a negative control reaction, an incubation with a boiled crude lentil extract was done. This reaction was allowed to continue for 6 h under the conditions mentioned above, leading to a 7% conversion of linoleic acid.

2.3. Analytical

Fatty acid methyl esters were analysed by SP-HPLC on a CP-Sphere Si column (Chrompack, 5 μm particles, 4.6 × 250 mm) using a HP1040A diode array UV detector (Hewlett Packard) and a HP7994A analytical workstation (Hewlett Packard) for data processing. Spectra of peaks were recorded at 2 nm resolution. Products were eluted isocratically with *n*-hexane/diethylether/HAc (90:10:0.1 v/v/v) [21] at a flow of 3.0 ml/min, which allows a complete separation of OXODs, HPODs and HODs in a single run. HAc was added to the eluent in order to be able to monitor unesterified products which might result from a partial esterification. Relative amounts of the different products were calculated from the peak-areas, assuming a molar absorbance of 22 000 at 268 nm for OXODs and 25 000 at 234 nm for HPODs and HODs. Peaks of 13-*ZE*-HOD and 9-*EZ*-HOD were collected for chiral analysis. 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 were purified for chiral analysis using the same SP-HPLC system.

The absolute configuration of the products was determined by chiral phase HPLC on a Chiralcel OD-R column (J.T. Baker). Products were eluted with MeOH/H_2O/HAc (85:15:0.1 v/v/v). In separate experiments the methyl esters of 13-*ZE*-HPOD and 9-*EZ*-HPOD were isolated from the esterified reaction products by the SP-HPLC method mentioned before and analysed without further derivatization by circular dichroism spectroscopy. CD-spectra (7–42 μM esterified HPOD in methanol, optical pathway 1.0 cm) were recorded on a JASCO J-600 spectropolarimeter. Spectra (200–270 nm, 1 nm steps) were accumulated for 20–40 min, were smoothed by averaging the data over 9 data points and corrected for an independently recorded baseline.

Mass spectra were obtained on a 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&W Scientific). OXOD methyl esters 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 hydroxy-derivatives. Part of the hydroxylinoleates were catalytically converted into hydroxystearates by bubbling hydrogen gas through a methanolic solution of the product in the presence of PtO_2. Hydroxystearates and hydroxylinoleates were converted into trimethylsilylethers by dissolving them in dry pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane (5:2:1, v/v). The resulting TMS derivatives were then analysed by GC-MS (see above, time program: 2 min at 140°C, then with 4°C/min to 280°C).

2.4. Electrophoresis and immunoblotting

SDS-PAGE [22] was performed on an SE-250 Mighty Small II vertical electrophoresis apparatus (Hoefer). IEF, with Ampholine pH 5–7 (Sigma) was performed on the same apparatus using the procedure of Robertson et al. [23] 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 h at 200 V followed by 1 h 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 [24]. 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. Marker proteins (Sigma) were rabbit muscle myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphory-

lase *b* (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), bovine erythrocyte carbonic anhydrase (29 kDa, p*I* 5.4 and 5.9), bovine milk β-lactoglobulin A (p*I* 5.1) and human erythrocyte carbonic anhydrase (p*I* 6.6). Densitometric analyses of Coomassie stained gels were performed on an LKB 2202 ultrascan laser densitometer.

2.5. Partial purification

All manipulations were performed at 4°C. Lentil seedling tissue (20 g) was homogenized in a Waring blender with 60 ml of extraction buffer (0.1 M sodium phosphate, 10 mM EDTA, 1% Triton X-100, pH 6.8). The homogenate was stirred for 30 min, filtered through a nylon gauze and centrifuged at 15 000 × *g* for 30 min. The supernatant was stirred for 90 min with 10% (w/v) Amberlite XAD-2 to reduce the Triton X-100 content to 0.1%. After filtration over a glass filter to remove the resin, the extract was dialysed against 3 changes of 1 l of column buffer (10 mM sodium phosphate, 1 mM EDTA, 0.1% Triton X-100, pH 6.8). The dialysed extract was centrifuged for 20 min at 15 000 × *g* and the supernatant was loaded on a DEAE-Sepharose (Pharmacia) column (2.0 × 20 cm) equilibrated with column buffer. LOX was eluted with a linear gradient of 0–1 M NaCl in 400 ml column buffer. Active fractions were pooled, brought to 20% glycerol (v/v) and stored at −80°C until further analysis.

3. Results

3.1. Developmental changes of LOX activity

LOX-activity in cotyledons, epicotyl and hypocotyl/radicle of germinating lentils was determined at 24 h intervals for 10 days and further at 48 h intervals until 18 days after germination (Fig. 1). In the cotyledons the LOX-activity declined throughout this period, whereas the LOX-activity in epicotyl and hypocotyl/radicle first increased and then declined. Highest LOX activities were found 5–6 days after germination, coinciding with the highest growth rate for the tissue. After 14 days little activity was left in any tissue.

3.2. Localization of LOX isoenzymes

The distribution of LOX in various parts of the seedlings was studied by dividing 5 day-old seedlings into 7 segments and assaying these segments for LOX activity (Fig. 2). The absolute amount of LOX in the cotyledons and in the tips of the shoots

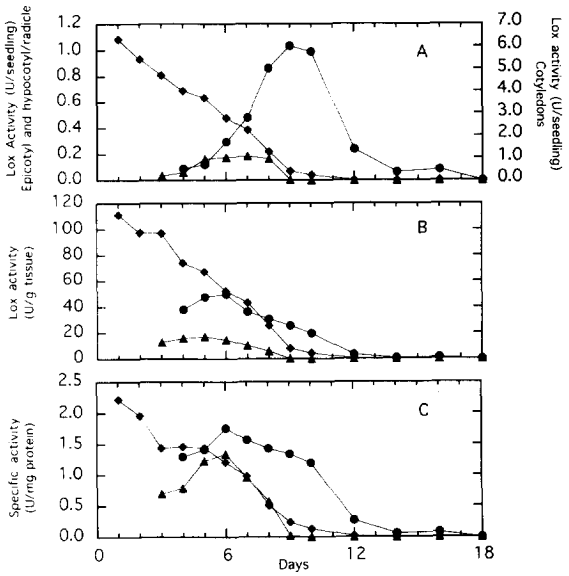

Fig. 1. Changes of LOX levels in the epicotyl (●), hypocotyl/radicle (▲) and cotyledons (◆) of developing lentil seedlings expressed as activity per seedling (A), per g (fresh weight) tissue (B) and per mg protein extracted (C). For each point the activity in three extracts from the same growth-experiment of seedlings was measured. The standard deviation for each point was less than 11%. Other growth-experiments of seedlings gave similar profiles.

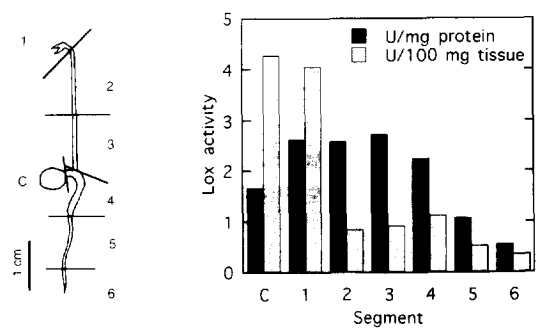

Fig. 2. Lipoxygenase activity in lentil seedling segments. Left: drawing indicating into which sections the 5 day-old seedlings were divided. Right: lipoxygenase activity assayed in those segments expressed as U/mg protein and as U/100 mg (fresh weight) seedling.

was high, but similar to values found in the rest of the epicotyl and in the hypocotyl when expressed as LOX-activity per mg protein. LOX-levels in the roots were low.

To see whether the LOX-activity in the newly developing tissues resulted from the appearance of new LOXs, not present in the cotyledons, extracts from the seedling segments and of seeds after 24 h of soaking were analysed with Western blots of SDS-PAGE and IEF gels, immunostained with antibodies against soybean LOX-1. Soaked seeds were used since they could be processed in an identical manner as seedling-parts. Preliminary experiments with extracts obtained with ground dry seeds showed that after 24 h of soaking no new LOXs had appeared in the cotyledons.

SDS-PAGE/Western analysis (Fig. 3) showed a single LOX band at 94 kDa for cotyledons and segments 5 and 6, whereas segments 1–4 also showed a 96 kDa LOX band. In the tip of the shoot the 96 kDa LOX dominated, whereas in the lower segments 94 kDa LOX was more abundant. We cannot exclude the possibility that the 94 kDa LOX originates from proteolysis of the 96 kDa LOX. The SDS-PAGE/Western blots showed no LOX bands lower than 94 kDa. Since the IEF/Western blots showed more bands then the SDS-PAGE/Western blots, the different bands on IEF/Western should represent different isoenzymes or isoforms of the same LOX-isoenzyme.

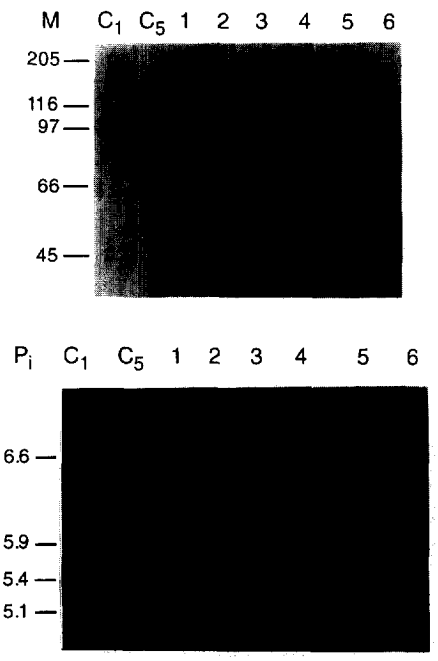

Fig. 3. SDS-PAGE/Western and IEF/Western analysis of lipoxygenase in different parts of lentil seedlings. SDS-PAGE (upper) and IEF (lower) gels were loaded with 0.05 units of lipoxygenase from the various lentil seedling segments (indicated above the lane, see Fig. 2) and immunodetected with antibodies to soybean LOX-1. Positions of marker-proteins are indicated on the left.

IEF was performed in a pH range of 5–7 since preliminary experiments showed no LOX outside this range. The IEF/Western-blot (Fig. 3) indicated the presence of four LOX-isoenzymes in the vegetative tissues, different from the LOXs in the cotyledons. A LOX with a p*I* of 6.3 was found in segments 3–6, of which segments 5 and 6 did not contain any other LOX. A LOX with a p*I* of 6.1 was found in segments 1–4. Two LOXs (p*I* 5.7 and 5.8) were major LOXs in segments 1 and 2 and minor LOXs in segments 3 and 4. The cotyledons contained several LOXs, with similar p*I* values (5.2–5.5). In the cotyledons of 5 day-old seedlings a new LOX appeared, not present in the seeds after 24 h of soaking. This LOX had the same p*I* (5.7) as one of the shoot-tip LOXs.

3.3. Partial purification

LOXs from extracts of cotyledons after 24 h of soaking, of the upper part of the epicotyl and of the hypocotyl/root of 5 day-old seedlings were partially purified using DEAE-chromatography (Table 1, Fig. 4). From the cotyledons two LOXs were obtained (C1 and C2), from the epicotyls three LOXs (E1, E2 and E3) and from the hypocotyl/root two LOXs (H1 and H2). Since the same column was used for all the purification experiments without repacking, identical LOXs in the different extracts should have eluted at roughly the same NaCl-concentration. Judged by this criterion E3 might be identical to C2.

SDS-PAGE/Western analysis of the fractions obtained (Fig. 5) showed that the partially purified LOXs were not proteolytically degraded. E2 appeared to be the 96 kDa LOX. IEF/Western blots showed that the LOXs had different p*I*s. For E3 we did not succeed in obtaining a p*I* value. Since

Table 1
Partial purification of lipoxygenases from lentils after 24 h soaking (C1 and C2) and from the epicotyl (E1, E2 and E3) or hypocotyl/radicle (H1 and H2) of 5 day-old seedlings

Lipoxygenase preparation	Total protein (mg)	Specific activity (U/mg protein)	Purification[a] (fold)	Yield[a] (%)	Purity[b] (%)
Cotyledon crude	660	0.45			
Fraction C1	31	0.96	2.1	10	45
Fraction C2	55	1.07	2.4	20	13
Epicotyl crude	162	1.8			
Fraction E1	12.1	5.7	3.2	24	22
Fraction E2	26.7	3.3	1.8	30	21
Fraction E3	21.3	0.5	0.27	4	7
Hypocotyl crude	73	0.39			
Fraction H1	1.3	4.1	10.5	19	27
Fraction H2	9.1	1.75	4.5	56	24

[a]Relative to the total amount of LOX in the crude extract.
[b]Estimated from densitometric analysis of Coomassie stained SDS-Page gels, assuming that the LOX-band on the gel was pure.

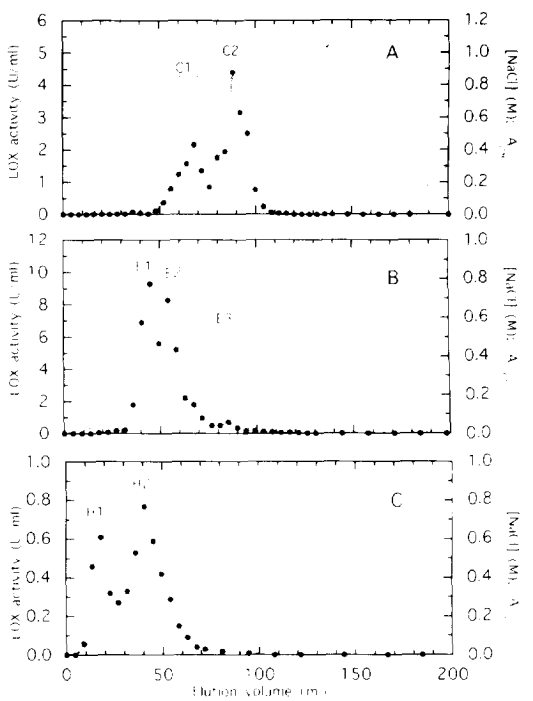

Fig. 4. DEAE-Sepharose chromatography of extracts of soaked lentils (A) and epicotyls (B) and hypocotyl/radicle (C) of 5 day-old lentil seedlings. Horizontal lines indicate the fractions pooled for further analysis. Plotted are LOX activity in the fractions (●), absorbance at 280 nm (——) and the NaCl concentration in the eluent (– – –).

it eluted from the column after E2 one can assume that its p*I* value must be below that of E2. C1 and C2 showed several bands, which might be caused by the presence of either microheterogeneities or of different isoenzymes. Because H2 showed two LOX-bands, and one of them had the same p*I* as H1, H2 is contaminated with H1. E1 and E2 appear to be contaminated with each other.

3.4. Characterization of LOX isoenzymes

The pH optima of the different LOXs were determined (Fig. 6). C1 showed 2 optima. This might be caused by the presence of two LOX isoenzymes in this fraction, one having an optimum at pH 9.0, the other at pH 6.5. The other fractions showed pH-optima around 6–6.5, with similar profiles. H2 was exceptional with a very broad optimum, centred at pH 5.75. Another property that distinguished the different LOX-isoenzymes was the amount of OXODs (characterized by an absorbance maximum at 285 nm) formed during the reaction. The ratio of HPOD/OXOD formation ranged from 97:3 for E3 to 54:46 for C1 at pH 6.5 (Table 2). The LOXs also showed different substrate preferences. Reaction rates with α-linolenic acid ranged from 17% (H2) to 115% (E2) of the rate with linoleic acid. Apart from not appearing

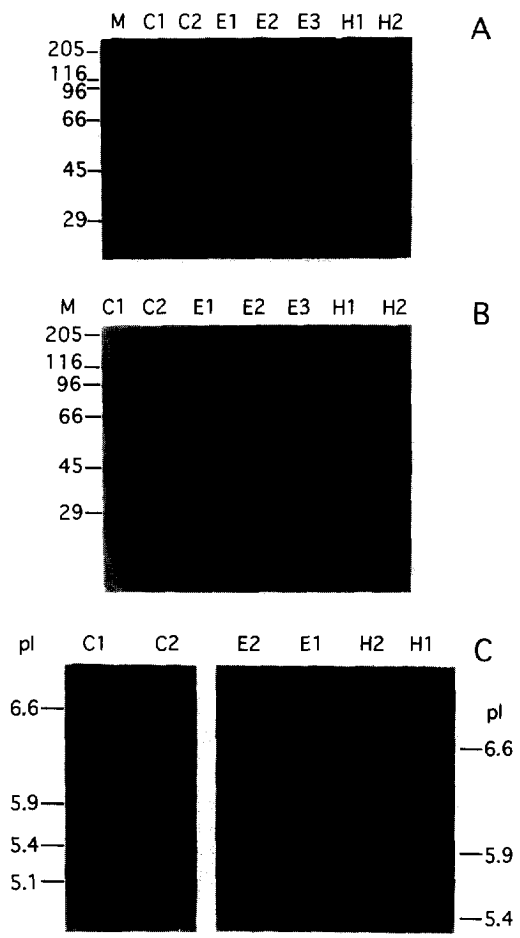

Fig. 5. Electrophoretic analysis of the different LOX-fractions obtained. (A) Coomassie Blue stained SDS-PAGE gel of the LOX-fractions. (B) Western blot of SDS-PAGE gel. (C) Western blots of IEF gels. Left blot: seed LOXs. Right blot: vegetative LOXs. Western-blots were immunostained with monoclonal antibodies to soybean LOX-1. Positions of molecular mass and p*I* markers are indicated.

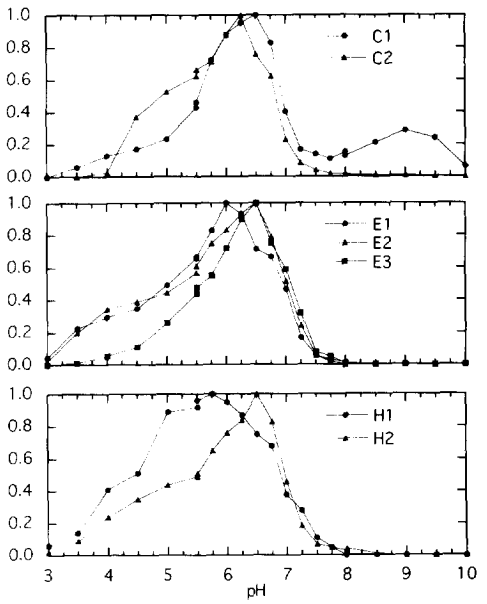

Fig. 6. pH Optima determined for the different LOX-fractions. Buffers used (0.1 M): pH 3–5.5, sodium acetate; pH 5.5–8, sodium phosphate; pH 8–10, sodium borate. Values are means of triplicates with a standard deviation of less than 8%.

on the IEF/Western blots, which might be caused by its high impurity, E3 did not differ in any property from C2, therefore it was probably identical to this isoenzyme.

3.5. Product analysis

Methyl esters of the reaction products obtained with linoleic acid from the different LOX-fractions were analysed by SP-HPLC. The chromatograms obtained (Fig. 7) showed 4 peaks (A–D) with an absorbance-maximum at about 268 nm, identified as 13-*EZ*-, 9-*ZE*-, 13-*EE*- and 9-*EE*-OXOD [25], respectively, followed by 8 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 [21], respectively. Unlike SP-HPLC methods based on hexane/isopropanol eluents [26,27], the hexane/diethylether eluent allowed complete separation of all OXOD, HPOD and HOD isomers.

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 [28]. *EE*-isomers of conjugated dienes have absorbance maxima at lower wavelengths than *EZ*- or *ZE*-isomers [29], and this was also found for the peaks assigned to these isomers (for OXODs 267 vs. 269 nm, for H(P)ODs 231 vs. 233 nm).

For the products of the reaction of linoleic acid with C1 at pH 6.5 we further analysed the different

Table 2
Characteristics of the different lentil LOX fractions

Fraction	Elution point[a] [NaCl] (M)	pH optimum	Ratio[b] H(P)OD/OXOD	Activity OT/OD[c] (%)	p*I*	Molecular mass (kDa)
C1	0.17	6.5[d]	78:22	98	5.4,5.5	94
		9.0[d]	54:46	65		
C2	0.22	6.5	96:4	38	5.2,5.3	94
E1	0.11	6.0	86:14	115	5.8	94
E2	0.13	6.5	85:15	112	5.7	96
E3	0.21	6.5	97:3	45	<5.7	94
H1	0.05	5.75	64:36	46	6.3	94
H2	0.10	6.5	58:42	17	6.1	94

[a]NaCl concentration at which the LOX-fraction eluted from the DEAE-Sepharose column.
[b]Molar ratio of H(P)OD/OXOD formation as determined by measuring enzyme activity at 234 and 285 nm.
[c]Relative activity towards α-linolenic acid (OT) compared with the activity towards linoleic acid (OD).
[d]C1 showed two optima. Enzyme characteristics were determined at both pHs.

peaks to confirm the assignments. Reduction of a particular 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 these products [27]. 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, and the fragment at *m/z* 311 to be more abundant than the fragment at *m/z* 225 for

Table 3
Analysis of the products from the reaction of the lentil LOX fractions with linoleic acid

Lipoxygenase	Products[a] (HPOD:HOD:OXOD)	Positional isomers[b,d] (13*ZE*:13*EE*:9*EZ*:9*EE*)		Optical isomers[c,d] (*R*:*S*)	
		HPOD	OXOD	13-*EZ*-HPOD	9-*ZE*-HPOD
Autoxidation	66:28:8	42:13:32:13	33:20:28:19	49:51 50:50	
Soy LOX1	94:5:1	85:1:13:1	77:5:14:4	24:76 (+)	6:94 (+)
C1, pH 9.0	76:19:5	28:4:62:6	36:16:42:6	29:71 (+)	65:35 (−)
C1	49:31:20	27:5:60:8	37:15:39:10	23:77 (+)	66:34 (−)
C2	66:29:5	76:5:13:6	49:20:18:12	9:91 (+)	46:54 (±)
E1	87:10:3	84:3:8:4	61:10:19:10	10:90 (+)	29:71 (+)
E2	82:10:9	61:7:23:9	33:20:30:17	17:83 (+)	33:67 (+)
E3	87:11:2	82:3:11:4	65:9:19:7	8:92 (+)	45:55 (±)
H1	70:14:16	33:10:46:12	40:17:33:10	28:72 (+)	52:48 (±)
H2	79:9:12	57:7:27:9	39:12:41:8	21:79 (+)	47:53 (±)

[a]Molar ratio of HPODs, HODs and OXODs as determined by SP-HPLC.
[b]Molar ratios of the 13-*EZ*, 13-*EE*, 9-*ZE* and 9-*EE* isomers as determined by SP-HPLC.
[c]Molar ratio of *R* and *S* optical isomer determined by CP-HPLC. Between brackets: sign of the Cotton-effect at 234 nm determined by CD-spectroscopy of the HPOD methyl ester. ±, indistinguishable from baseline.
[d]The distribution of positional and optical isomers for the HODs obtained was almost identical to those for the HPODs.

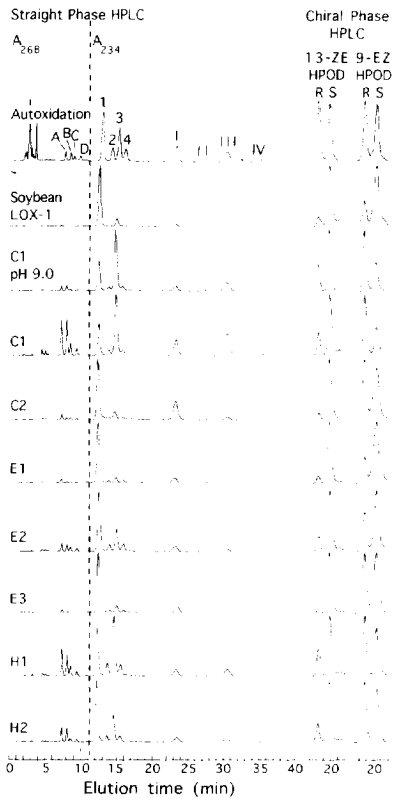

Fig. 7. HPLC analysis of esterified products obtained from linoleic acid with the different lipoxygenases. Auto: autoxidation. Incubations were done at pH 6.5 unless otherwise indicated. For the SP-HPLC chromatograms absorbance at 268 nm is shown for the first 11 min, the absorbance at 234 nm thereafter (transition indicated by vertical dashed line). Chromatograms were normalized to the height of the largest peak. 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. HPODs were reduced to HODs for CP-HPLC.

the 13-products, whereas the reverse was found for the 9-products [30].

CP-HPLC on a Chiralcel OD-R column afforded separation of *R* and *S* enantiomers of esterified 13-*ZE*-HOD and 9-*EZ*-HOD (Fig. 7). The separation of *EE*-HOD enantiomers on this column was not possible. Since all products eluted at approximately the same time, it was essential to separate the different reaction products before CP-HPLC. This was achieved by the SP-HPLC system mentioned before. Enantiomers were identified by comparison with the products obtained with soybean LOX-1 at pH 6.5, yielding *S* enantiomers of 13-*ZE*-HPOD and 9-*EZ*-HPOD [28]. The identification was independently confirmed by CD-spectra of the 9-*EZ*-HPOD and 13-*ZE*-HPOD obtained with the different LOXs. Although the spectra did not afford a quantitative assessment, they could be interpreted in terms of a positive or negative Cotton-effect at 234 nm.

The distribution of the different products, isomers and enantiomers calculated from the HPLC-analyses is shown in Table 3. In all cases, the isomer and enantiomer distribution for HODs (not shown) was nearly identical to those found for HPODs.

4. Discussion

This work describes the developmental changes of LOX in germinating lentils and shows that the LOX activity in the new tissues results from the appearance of new LOXs, not present before in the seeds.

During germination the LOX activity in lentil shows a pattern often found in plants [6–9]: a continuous decline in the cotyledons and a burst of LOX-activity in the developing new tissues, reaching a maximum that coincides with the maximum growth rate of the tissue. The experiments point to the presence of at least two different LOXs (C1 and C2) in the seeds and four (E1, E2, H1 and H2) in the newly developing tissues of the seedlings. The latter are distributed over the seedling in a tissue specific manner. E1 and E2 are specific for the tip of the shoot, H2 is found mainly in the lower part of the epicotyl and in the hypocotyl and H1 is found in the hypocotyl and the root. The different LOXs not only differ in electrophoretic properties, but have also different enzymatic properties. Therefore, the different LOXs found are genuine isoenzymes and not isoforms of the same enzyme.

Like in lentil, the seeds of other legumes such as chickpea (*Cicer arietinum*), pea (*Pisum sativum*) and kidney bean (*Phaseolus vulgaris*) contain two major LOXs. One similar to C1, producing a slight excess of 9-*EZ*-HPOD over 13-*ZE*-HPOD, accom-

panied by OXODs, and another LOX, which has a lower p*I*, synthesizing 13-*ZE*-HPOD, like C2 [25,27,31,32]. The enantiospecificity of C1, which synthesizes 9-(*R*)-*EZ*-HPOD and 13-(*S*)-*ZE*-HPOD, resembles the type II LOXs of soybean and pea [33]. Type II LOX of soybean consists of several isoenzymes [34]. For C1 we found two bands in the IEF-analysis and measured two pH optima, so it is possible that C1 consists of two isoenzymes. In a previous study, non-denaturing gel electrophoresis indicated the presence of three LOXs in lentil-seeds [18]. These lentil seed LOXs all had a p*I* of 5.3. This value from an IEF gel of pH 3–10 closely resembles our values. The pattern of at least two major LOXs in the legume seeds contrasts with the cereals. The seeds of wheat [32], barley [35], maize [36] and rice [6] all contain one major LOX, which synthesizes only 9-(*S*)-*EZ*-HPOD from linoleic acid.

In soybean, which is the main model system for plant LOX research, new LOXs appear during germination. In the cotyledons three new LOXs appear (L4, L5 and L6) [37]. L4 is an unspecific LOX, which synthesizes 9-*EZ*-HPOD and 13-*ZE*-HPOD, like H1, whereas L5 and L6 synthesize 13-*ZE*-HPOD, like E1, E2 and H2. Transcripts corresponding to L4 are found in all soybean seedling tissues, whereas L6 transcripts are concentrated in the hook and the elongation regions of the hypocotyl/radicle [38]. IEF analyses of extracts from the hypocotyl/radicle of soybean seedlings indicate the presence of LOXs with p*I*s of 5.1, 5.3 and 5.9 [39,40]. Until now no correlation has been made between these IEF-bands and enzymatic properties of soybean seedling LOXs, making it difficult to compare the lentil seedling LOXs with soybean LOXs. Young leaves of 1 month old soybean plants contain several LOX isoenzymes, which can be divided into acidic LOXs with p*I*s around 5.1 and neutral LOXs with p*I*s around 7.0 [10,40]. These types of LOXs appear to be absent in lentil seedlings.

Pea seedlings contain at least 5 LOXs, a number similar to that found in lentil, with different isoenzymes in seeds, stems and leaves, but no attempt was made to characterize these LOXs [41]. Eiben and Slusarenko [13] did a thorough analysis of LOX in various parts of French bean (*Phaseolus vulgaris*), at different plant ages. They differentiated between type I activity, defined by HPOD formation at pH 9.0, and type II activity, defined by OXOD formation at pH 6.5. They measured more OXOD forming LOX in the hypocotyl than in the stem or primary leaves, which is similar to the situation in lentil seedlings. From immunostained tissue-prints they found a decrease in LOX-activity from the top downwards in the seedling, which agrees with our results.

The main conclusion we infer from this work is that lentil seeds contain the same types of LOX-isoenzymes as other legume-seeds and that during germination new LOXs appear in the newly developing tissues, clearly differing in enzymatic and physical properties from the LOXs already present in the seeds, and that these vegetative LOXs emerge in a tissue specific manner.

Acknowledgements

This research was supported by European Community grant SC1-0197. We wish to thank Dr. Mauro Maccarrone (University of Rome 'Tor Vergata') for the donation of antibodies to soybean lipoxygenase-1 and Mr. Guus van Zadelhoff for assisting with the HPLC analyses.

References

[1] J.N. Siedow, Plant lipoxygenase: structure and function. Annu. Rev. Plant Physiol. Plant Mol. Biol., 42 (1991) 145–188.

[2] S. Yamamoto, Mammalian lipoxygenases — molecular structures and functions. Biochim. Biophys. Acta, 1128 (1992) 117–131.

[3] G. Shechter and S. Grossman, Lipoxygenase from baker's yeast: purification and properties. Int. J. Biochem., 15 (1983) 1295–1304.

[4] D.C. Zimmerman and B.A. Vick, Lipoxygenase in *Chlorella pyrenoidosa*. Lipids, 8 (1973) 264–266.

[5] D. Iny, A. Pinsky, M. Cojocoru and S. Grossman, Lipoxygenase of *Thermoactinomyces-vulgaris*, purification and characterization of reaction products. Int. J. Biochem., 25 (1993) 1313–1323.

[6] H. Ohta, S. Ida, B. Mikami and Y. Morita, Changes in lipoxygenase components of rice seedlings during germination. Plant Cell Physiol., 27 (1986) 911–918.

[7] P.L. Guss, V. Macko, T. Richardson and M.A. Stahmann, Lipoxidase in early growth of wheat. Plant Cell Physiol., 9 (1968) 415–422.

[8] J.-L. Beneytout, A. Najid and M. Tixier, Changes in lipoxygenase activity during seedling development of *Lupinus albus*. Plant Sci., 58 (1988) 35–41.

[9] K. Matsui, T. Kajiwara, K. Hayashi and A. Hatanaka, Tissue specific heterogeneity of lipoxygenase in cucumber seedlings. Agric. Biol. Chem., 52 (1988) 3219–3221.

[10] W.S. Grayburn, G.R. Schneider, T.R. Hamilton-Kemp, G. Bookjans, K. Ali and D.F. Hildebrand, Soybean leaves contain multiple lipoxygenases. Plant Physiol., 95 (1991) 1214–1218.

[11] A. Pinsky, S. Grossmann and M. Trop, Lipoxygenase content and antioxidant activity of some fruits and vegetables. J. Food Sci., 36 (1971) 571–572.

[12] A. Pinsky, J. Sporn, S. Grossman and M. Rivlin, Lipoxygenase isoenzymes from *Solanum tuberosum*. Phytochemistry, 12 (1973) 1051–1055.

[13] H.G. Eiben and A.J. Slusarenko, Complex spatial and temporal expression of lipoxygenase genes during *Phaseolus-vulgaris* (L.) development. Plant J., 5 (1994) 123–135.

[14] D.F. Hildebrand, J.G. Rodriguez, C.S. Legg, G.C. Brown and G. Bookjans, The effects of wounding and mite infestation on soybean leaf lipoxygenase levels. Z. Naturforsch. C, 44 (1989) 655–659.

[15] B.A. Vick and D.C. Zimmerman, Oxidative systems for modification of fatty acids: the lipoxygenase pathway, in: P.K. Stumpf and E.E. Conn (Eds.), The Biochemistry of Plants. A Comprehensive Treatise, Academic press, New York, 1987, pp. 55–90.

[16] J.A. Blain, J.J. Doherty and J.P. Todd, Lentil lipoxidase. Chem. Ind. (London), 10 (1959) 1216–1217.

[17] P.R.Q. Chang and A.R. McCurdy, Lipoxygenase activity in fourteen legumes. Can. Inst. Food Sci. Technol. J., 18 (1985) 94–96.

[18] N.A.M. Eskin and H.M. Henderson, A study of lipoxygenase from lentils and lupins. Ann. Technol. Agric., 26 (1977) 139–149.

[19] B. Axelrod, T.M. Cheesbrough and S. Laakso, Lipoxygenase from soybeans. Methods Enzymol., 71 (1981) 441–451.

[20] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, B.J. Olson and D.C. Klenk, Measurement of protein using bicinchoninic acid. Anal. Biochem., 150 (1985) 76–85.

[21] J.I. Teng and L.L. Smith, High performance liquid chromatography of linoleic acid hydroperoxides and their corresponding alcohol derivatives. J. Chromatogr., 350 (1985) 445–451.

[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227 (1970) 680–685.

[23] E.F. Robertson, H.K. Dannelly, P.J. Malloy and H.C. Reeves, Rapid isoelectric focusing in a vertical polyacrylamide minigel system. Anal. Biochem., 167 (1987) 290–294.

[24] M. Maccarrone, G.A. Veldink and J.F.G. Vliegenthart, Inhibition of lipoxygenase activity in lentil protoplasts by monoclonal antibodies introduced into the cells via electroporation. Eur. J. Biochem., 205 (1992) 995–1001.

[25] L.C. Sanz, A.G. Perez, J.J. Rios and J.M. Olias, Positional specificity of ketodienes from linoleic acid aerobically formed by lipoxygenase isozymes from kidney bean and pea. J. Agric. Food Chem., 41 (1993) 696–699.

[26] Z. Wu, D.S. Robinson, C. Domoney and R. Casey, High-performance liquid chromatography analysis of the products of linoleic acid oxidation catalyzed by pea (*Pisum sativum*) seed lipoxygenases. J. Agric. Food Chem., 43 (1995) 337–342.

[27] H. Kühn, R. Wiesner, J. Rathmann and T. Schewe, Formation of ketodienoic fatty acids by the pure pea lipoxygenase-1. Eicosanoids, 4 (1991) 9–14.

[28] H.W. Gardner, Soybean lipoxygenase-1 enzymatically forms both (9*S*)- and (13*S*)-hydroperoxides from linoleic acid by a pH-dependent mechanism. Biochim. Biophys. Acta, 1001 (1989) 274–281.

[29] A. Lezerovich, Derivative UV spectra of lipid conjugated dienes. J. Am. Oil Chem. Soc., 63 (1986) 883–888.

[30] W.C. Hubbard, A.J. Hough Jr, A.R. Brash, J.T. Watson and J.A. Oates, Metabolism of linoleic and arachidonic acids in vx^{TM} carcinoma tissue: identification of monohydroxy octadecadienoic acids and monohydroxy eicosatetraenoic acids. Prostaglandins, 20 (1980) 431–447.

[31] L.C. Sanz, A.G. Perez, J.J. Rios and J.M. Olias, Physicochemical properties of chickpea lipoxygenases. Phytochemistry, 32 (1992) 3381–3384.

[32] H. Kühn, R. Wiesner, V.Z. Lankin, A. Nekrasov, L. Alder and T. Schewe, Analysis of the stereochemistry of lipoxygenase-derived hydropolyenoic fatty acids by means of chiral phase high-pressure liquid chromatography. Anal. Biochem., 160 (1987) 24–34.

[33] C.P.A. Van Os, G.P.M. Rijke-Schilder and J.F.G. Vliegenthart, 9-L_R-linoleyl hydroperoxide, a novel product from the oxygenation of linoleic acid by type-2 lipoxygenases from soybeans and peas. Biochim. Biophys. Acta, 575 (1979) 479–484.

[34] M.C. Feiters, G.A. Veldink and J.F.G. Vliegenthart, Heterogeneity of soybean lipoxygenase-2. Biochim. Biophys. Acta, 870 (1986) 367–371.

[35] P.G.M. Van Aarle, M.M.J. De Barse, G.A. Veldink and J.F.G. Vliegenthart, Purification of a lipoxygenase from ungerminated barley. Characterization and product formation. FEBS Lett., 280 (1991) 159–162.

[36] E. Poca, H. Rabinovitch-Chable, J. Cook-Moreau, M. Pagés and M. Rigaud, Lipoxygenases from *Zea mays* L. Purification and physicochemical characteristics. Biochim. Biophys. Acta, 1045 (1990) 107–114.

[37] T. Kato, H. Ohta, K. Tanaka and D. Shibata, Appearance of new lipoxygenases in soybean cotyledons after germination and evidence for expression of a major new lipoxygenase gene. Plant Physiol., 98 (1992) 324–330.

[38] T.K. Park, M.A. Holland, J.G. Laskey and J.C. Polacco, Germination-associated lipoxygenase transcripts persist in maturing soybean plants and are induced by jasmonate. Plant Sci., 96 (1994) 109–117.

[39] T.K. Park and J.C. Polacco, Distinct lipoxygenase species appear in the hypocotyl/radicle of germinating soybean. Plant Physiol., 90 (1989) 285–290.

[40] D.M. Saravitz and J.N. Siedow, The lipoxygenase isozymes in soybean (*Glycine max* (L.) Merr.) leaves. Plant Physiol., 107 (1995) 535–543.

[41] P.J.P. Anstis and J. Friend, The isoenzyme distribution of etiolated pea seedling lipoxygenase. Planta, 115 (1974) 329–335.