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LIPOXYGENASE ISOENZYMES OF FRENCH BEAN PERICARP: SEPARATION, CHARACTERIZATION AND CHLOROPHYLL-BLEACHING ACTIVITY

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Key Word Index—*Phaseolus vulgaris*; Leguminosae, french bean, lipoxygenase isoenzymes, chromatofocusing; chlorophyll-bleaching

Abstract—Two lipoxygenase isoenzymes, Lα and Lβ, were partially purified from pericarp of french bean. The two isoenzymes differed in pI, M_r, apparent K_M and lag phase Both could be categorized as type 2 lipoxygenase, based on their acidic optimum pH, simultaneous production of carbonyl compounds and hydroperoxides in the presence of oxygen, aerobic chlorophyll-bleaching activity and inhibition by chlorophyll *a* In the presence of a sufficient amount of preformed hydroperoxides, it was possible to show competitive inhibition of Lα and Lβ by chlorophyll *a*

INTRODUCTION

In the presence of molecular oxygen, lipoxygenase (linoleate = O_2 oxidoreductase EC 1.13.11.12) catalyses the oxidation of C_{18}-unsaturated fatty acids with a *cis,cis*-1,4 pentadiene group to give hydroperoxides as primary products. In the course of the reaction oxodienoic acids, absorbing at 285 nm [1], may, depending on the reaction conditions and the lipoxygenase type, be formed. Lipoxygenase isoenzymes are categorized as type 1 or type 2 depending on their pH optimum and product specificity The type 1 lipoxygenase has optimum activity around pH 9 while the type 2 lipoxygenase is generally considered to have a more acidic pH optimum (pH 5–7) [2] It has been demonstrated that anaerobic conditions are required for the secondary production of oxodienoic acids catalysed by some lipoxygenases such as soybean L1 [1, 3] and lipoxygenase b from seeds of bush beans [4] whereas oxygen is required for their production by soybean type 2 lipoxygenases [5] and lipoxygenase a from bush beans [4].

Changes in pigments and colour during the storage of frozen unblanched vegetables have been associated with the oxidation of unsaturated fatty acids Reports have implicated lipoxygenase in the bleaching of plant carotenoids and chlorophylls during fatty acid oxidation [2, 6–11]. However, lipoxygenase isoenzymes differ in their co-oxidation activity. Thus, the carotene oxidase activity of soybean is associated with the type 2 lipoxygenases only [2, 12].

The aim of this study was to isolate and characterize lipoxygenase constituents in french bean pericarp in order to compare their eventual chlorophyll-bleaching activity. Lipoxygenase isoforms were characterized by pH optima, K_M, carbonyl forming ability, isoelectric point and M_r.

RESULTS AND DISCUSSION

Detection of lipoxygenase activity in gel

After PAGE, lipoxygenase activity was detected by two methods In the first, the method of Verhue and Francke [13] used for soya lipoxygenase was modified to allow the identification of french bean lipoxygenases after pore gradient electrophoresis It was found that ethanol and the dye *o*-dianisidine are both inhibitors of french bean lipoxygenases The poor solubility of linoleic acid below pH 9 is well known In the modified method, the ethanol concentration was reduced, the linoleic acid concentration was increased in order to be close to saturation and *o*-dianisidine was added after preincubation with the substrate Under these conditions, the orange colour appeared after about 48 hr The second method (slicing of the gels into 5 mm segments and enzyme assay of each segment) was successful for isoelectric focusing and pore gradient electrophoresis gels

Partial purification of the lipoxygenase isoforms

Two lipoxygenase isoforms were separated from french bean pericarps by chromatofocusing after ammonium sulphate fractionation and dialysis The elution profile on chromatofocusing is shown in Fig 1 Two forms of lipoxygenase were eluted successively from the column, Lα (average elution pH of 6 6) and Lβ (average elution pH of 5.4) The absorbance profile at 280 nm indicates that two major peaks of protein without enzyme activity are removed during this step. The results of the partial purification are summarized in Table 1 The better purification factor obtained for Lα (31-fold) in comparison to Lβ (9-fold) was confirmed by isoelectric focusing (Fig. 2). Four bands are visible under these conditions for Lα and

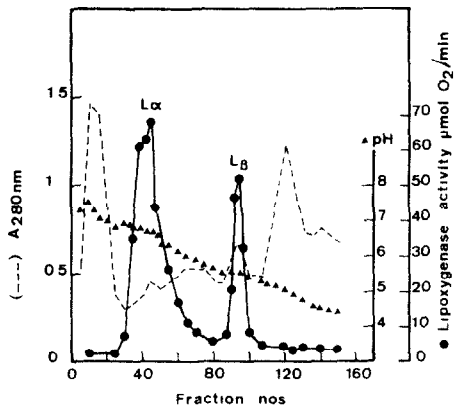

Fig 1 Separation of french bean lipoxygenase isoenzymes on a chromatofocusing column (60 × 1 2 cm) Flow rate was regulated to 22 ml/hr and 3 ml fractions were collected ---, Protein absorbance at 280 nm, ●— —●, lipoxygenase activity measured by polarography at pH 6 35 with 1 33 mM linoleic acid and 30 μl of each fraction in 3 9 ml total volume. ▲ ▲, pH

it is very likely that at least the main band (arrow, pI 7 3) and probably the minor band above corresponds to lipoxygenase For Lβ, the most basic constituent (arrow, pI 6.6) is lipoxygenase, but several bands without detectable enzymatic activity are visible on the diagram As with most of the lipoxygenases characterized in plants [14], the enzyme of french bean pericarps appears to be polymorphic The major form, Lα, which can be constituted of two isoforms, seems nearly pure but the minor enzyme, Lβ is contaminated by several proteins

Properties of Lα and Lβ

M_r determinations in denaturating (SDS-PAGE) and native (pore-gradient electrophoresis) conditions were compared The results of pore gradient electrophoresis after protein staining are shown in Fig. 3. After slicing of the gels, enzyme activity was detected in 3 and 2 consecutive segments for Lα and Lβ respectively (Fig 3), suggesting the presence of more than one constituent, at least for Lα By *o*-dianisidine staining, 2 bands (arrows) were detected for Lα (M_r 290 000 and 240 000) and 1 band

Table 1 Partial purification of lipoxygenases Lα and Lβ (lipoxygenase activity was measured by spectrophotometry at 234 nm)

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification relative to crude enzyme
Crude extract	2160	381	0 176	100	1
20–55% $(NH_4)_2SO_4$	247	190 4	0 771	50	4 3
Chromatofocusing pooled fractions					
Lα (nos 37 49)	5 09	27 8	5 47	7 2	31
Lβ (nos 90 99)	7 21	11 4	1 58	3	9

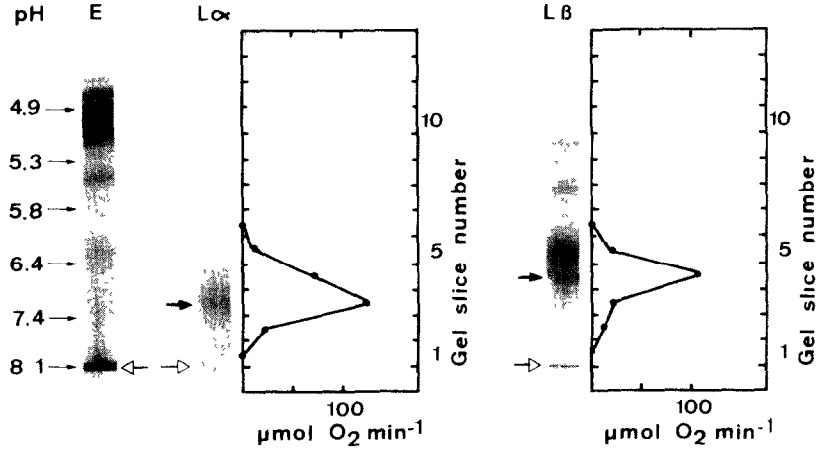

Fig 2 Isoelectric focusing (pH 3–10) of crude extract (E) and of Lα and Lβ fractions obtained after chromatofocusing The pooled fractions were concentrated 7 5- and 8 5-fold for Lα and Lβ respectively After electrophoresis, part of the gel was stained with Serva Violet 49 (electrophoregrams) For Lα and Lβ, the other part of the gel was sliced into segments, from the sample application, and assayed for enzyme activity by polarography (curves) White arrows: samples applications Positions of stained isoelectric points markers (BDH) are indicated on the left of the figure

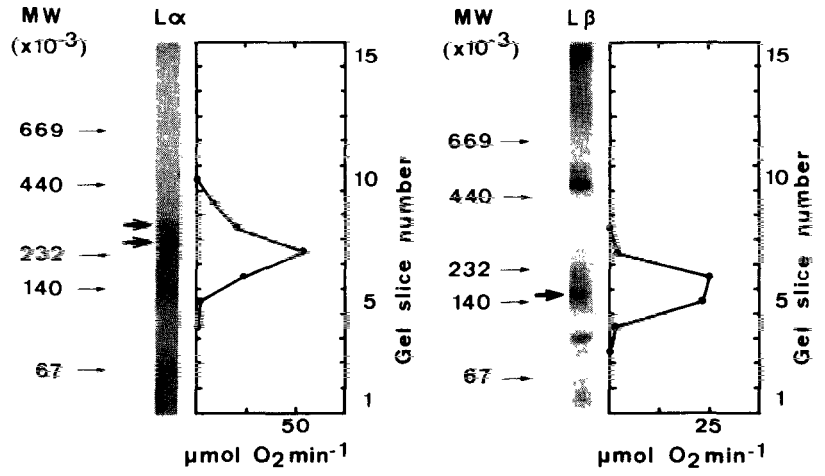

Fig 3 Gradient pore electrophoresis (4–30% acrylamide) of Lα and Lβ fractions obtained after chromatofocusing The pooled fractions were concentrated 9- and 8 5-fold for Lα and Lβ respectively After electrophoresis, part of the gel was stained with Coomassie Brilliant Blue R-250 (electrophoregrams) The other part of the gel was sliced into segments and assayed for enzyme activity by polarography (curves) Positions of M_r markers (high M_r calibration kit from Pharmacia) are indicated on the left of each diagram

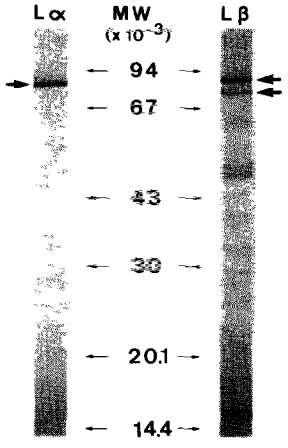

Fig 4 SDS-PAGE of Lα and Lβ fractions obtained after chromatofocusing The pooled fractions were TCA precipitated and 12 μg of proteins were applied on each lane Positions of M_r markers (low M_r calibration kit from Pharmacia) is indicated on the middle of the figure

(arrow) in the case of Lβ (M_r 160 000) By SDS-PAGE in reducing conditions (Fig. 4), all the M_rs of constituents detected were below 90 000. The M_r of the main band visible for Lα, which is likely to correspond to lipoxygenase, was 85 000. However, a similar band was not observed for Lβ (the M_r of the two bands visible in this part of the gel were 89 000 and 80 000 respectively). Together, these results suggested an oligomeric structure from different polypeptide chains for Lα and Lβ However, an *in vitro* aggregation of the enzyme cannot be excluded and more studies are necessary to confirm the structure suggested here for french bean pericarp enzymes. The high M_rs observed for Lα and Lβ are quite unusual Most of the M_rs of plant lipoxygenases fall between 70 000 and 120 000 However, M_rs of 250 000 and 240 000 were reported for sunflower seeds [15] and watermelon [16] enzymes respectively On the other hand, oligomeric structure was reported for the enzymes of tomato [17] and watermelon [16], but in most studies, the M_r was not determined in denaturating conditions. Clear evidence was not obtained for the soybean enzyme with some results indicating one polypeptide chain only [18, 19] and others oligomeric structures [20–22].

The lipoxygenases Lα and Lβ exhibited a narrow range of pH activity with a maximum at 6.35. The two isoenzymes have no activity below pH 5 5 or above pH 7

Lα and Lβ differ in their K_M values as determined by a Lineweaver–Burk plot. Apparent K_M (linoleic acid) values of 0.3 mM and 1.9 mM were obtained for Lα and Lβ respectively when activities were measured between 0 05 mM and 1 mM linoleic acid in air saturated solutions at pH 6.3. It is noteworthy that K_M values differ markedly according to the substrate concentration range chosen. For example, K_M (linoleic acid) values of 2 74 and 4 27 mM were determined respectively for Lα and Lβ when the substrate concentrations were between 0.2 and 10 mM (Hanes method). Similar discrepancies between the K_M values determined by different authors for soybean lipoxygenase 1 have been noted [19] and are probably due to the poor solubilization of linoleic acid at high concentrations [23].

Hydroperoxide formation (A_{234}) and carbonyl compound production (A_{285}) with time were compared for Lα and Lβ in conditions giving a similar rate of oxygen consumption (Fig. 5). Hydroperoxide and carbonyl compound formation began simultaneously in both cases. When oxygen is depleted, hydroperoxide formation stops and carbonyl compound production continues slowly. Thus, Lα and Lβ can be both categorized as type 2 like most plant lipoxygenases, according to their acidic pH optima and their ability to produce simultaneously hydroperoxide and carbonyl compounds when oxygen is present. The inability to produce carbonyl compounds

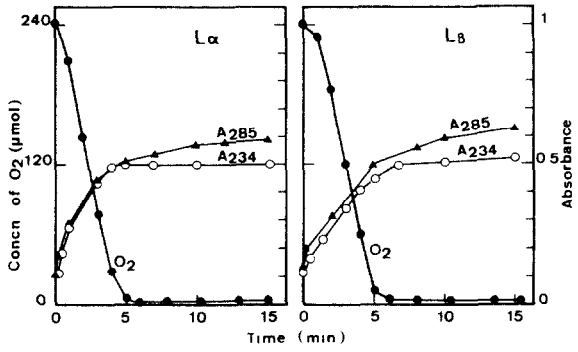

Fig 5 Formation of hydroperoxide (A_{234}) and carbonyl products (A_{285}) from linoleic acid catalysed by lipoxygenases Lα and Lβ in a closed system The reaction mixtures were air-saturated at 25° and contained 1 62 mM linoleic acid and 0 1 M phosphate–citrate buffer, pH 6 35 For the same rate of oxygen uptake measured by polarography, 60 μl of Lα and 200 μl of Lβ were added in the reaction mixture (3 920 ml) Hydroperoxide formation was measured at 234 nm in a 1 mm wide cuvette. A_{234} must be multiplied by 10

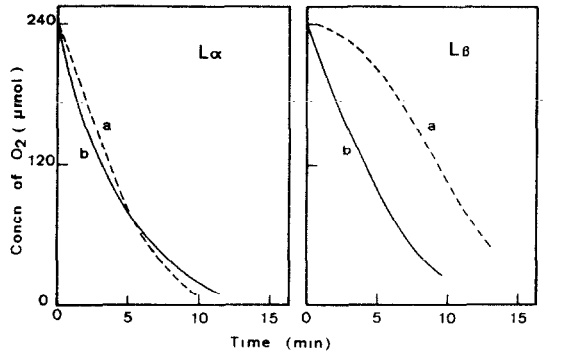

Fig 6 Progress curves for oxygen uptake during 2 mM linoleic acid oxidation catalysed by french bean lipoxygenases Lα and Lβ Fresh linoleic acid soln containing 0 05 mM of hydroperoxides (curves a) and oxidized linoleic acid soln containing 0 2 mM of hydroperoxides (curves b) were used The reaction was initiated with 30 μl of Lα or 180 μl of Lβ in 3 9 ml reaction mixture pH 6 35

until depletion of the dissolved O_2 has been reported only for lipoxygenases isolated from seeds, like pea [24], soybean [1], bush bean [4] and broad bean [25]

Differences between Lα and Lβ were observed by comparing the lipoxygenase reaction progress curves in relation to the oxidation of linoleic acid (Fig. 6). With fresh linoleic acid solution (2 5% of linoleic acid converted into hydroperoxides), Lβ had a lag period (Fig 6, curve a) while with Lα the initial velocity can be obtained immediately after the addition of the enzyme to the reaction mixture (Fig 6, curve a) When oxidized linoleic acid solution (10% of linoleic acid converted into hydroperoxides) was used as substrate, no lag period appears for Lβ and the initial velocity is speeded up for Lα (curves b, Fig. 6) Lag phases suppressible by adding hydroperoxides have already been observed [26-28] From this activation of the enzyme by its own product, Smith and Lands [29] and Garssen [26] concluded that the enzyme has a product binding site This site would be a regulator site on which the product or the substrate would bind [30] Our results show that hydroperoxides requirement to remove the lag period differ markedly according to the isoenzymes and is one of its enzymatic properties

Chlorophyll a bleaching and effects of pigment concentration on linoleate oxidation

In the presence of oxygen, lipoxygenases Lα and Lβ are good chlorophyll bleachers and have very weak anaerobic activity (Table 2) resembling in this respect soybean type 2 isoenzyme [5] For both Lα and Lβ, the bleaching activity was stimulated by hydroperoxides present in oxidized linoleic acid solution (Fig 7) An increased bleaching was also observed [5] when hydroperoxides were produced by incubating soybean type 2 lipoxygenase with linoleic acid before adding the chlorophyll *a* The same result was observed for co-oxidation of canthaxanthine [31] which was stimulated by addition of hydroperoxides These findings show that hydroperoxides act indirectly on pigment bleaching by activation of the lipoxygenase [31]

Chlorophyll *a* strongly inhibited the oxidative activity of Lα and Lβ on linoleic acid However, a Dixon plot of the effects of chlorophyll on the reaction do not allow the determination of the inhibition type when fresh linoleic acid solution is used (data not shown) Discontinuous lines were observed, for Lα and Lβ, as reported by Cohen *et al* [5] for soybean lipoxygenase-2, suggesting an additional inhibitory effect for weak lipoxygenase activities. When Lα and Lβ were markedly inhibited (high chlorophyll a concentration) long lag phases appear after which full activity cannot be measured This observation is probably related to the 'self-catalysed destruction' of the enzyme noted by Smith and Lands [32] and Cook and Lands [33] A typical Dixon plot was obtained with oxidized linoleic acid solution (Fig 8) showing clearly the competitive inhibition of Lα ($K_I = 1\ 75$ μmol) and Lβ ($K_I = 0\ 76$ μmol) by chlorophyll *a* This result indicated the need to provide a sufficient amount of preformed hydroperoxides if significant enzymatic activity is to be measured in the presence of an inhibitor

Table 2 Chlorophyll *a* bleaching by lipoxygenases Lα and Lβ under aerobic and anaerobic conditions

	Chlorophyll *a* bleaching (ΔA_{668}/10 min)	
Initial $A_{668} = 0\ 210$	Aerobic (air)	Anaerobic (N_2)
Lα	0 162	0 028
Lβ	0 127	0 025

Concentrations in the reaction mixture were 1 33 mM linoleate, 0 037% Tween 80 in 0 1 M Phosphate-citrate buffer, pH 6 35 The reaction was started by addition of 200 μl enzyme in 30 ml total volume

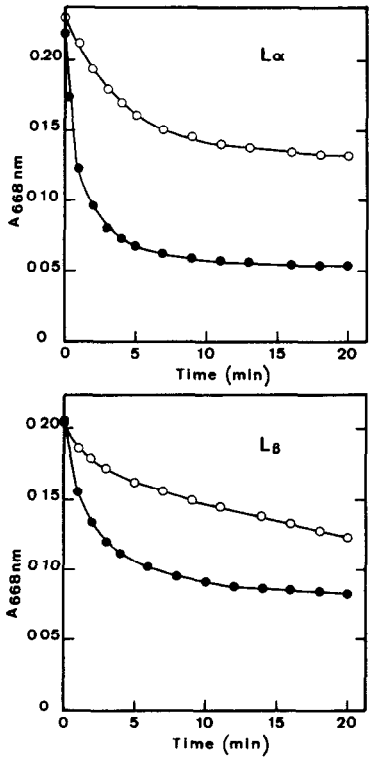

Fig. 7 Bleaching of chlorophyll *a* by lipoxygenases Lα and Lβ in a closed system at pH 6 35 Reaction mixture contained 4 5 μmol chlorophyll *a*/ml, and 30 μl of Lα or 120 μl of Lβ in 3.9 ml total vol o——o, fresh soln of 2 mM linoleic acid, ●——●, oxidized soln of 2 mM linoleic acid

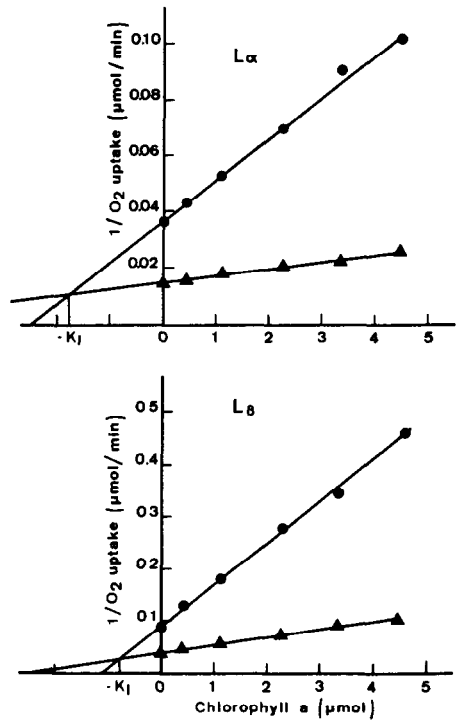

Fig 8 Dixon plots of lipoxygenase activity of Lα and Lβ in presence of different concentrations of chlorophyll *a*. ●——●, 2 mM linoleic acid, ▲——▲, 10 mM linoleic acid Lipoxygenase activity was measured by polarography at pH 6 35

CONCLUSION

The results presented here are a first characterization of lipoxygenase isoenzymes in french bean pericarp Two enzymes or enzyme groups were separated, differing by several properties. Both enzymes are good chlorophyll bleachers in the presence of oxygen and can be categorized as type 2 lipoxygenase. No type 1 lipoxygenase could be demonstrated in french bean pericarp, in contrast to the identification of type 1 and type 2 enzymes in bush bean seeds [4]. It is tempting to speculate that type 1 lipoxygenase is specifically present in seeds The confirmation of this specificity would be an interesting point in the understanding of the physiological role of lipoxygenases in plants.

EXPERIMENTAL

Materials. French beans (*Phaseolus vulgaris* L., cv rugallise) were harvested from C N R A , Versailles (France). The pod had a 13% dry matter and a seed/pod ratio of 21% After harvest the beans were frozen and stored at −65°

Extraction and separation of the lipoxygenase isoforms. Seeds and pericarps were separated before total thawing Pericarps (190 *g*) were immediately ground in liquid N_2 for 5 min The powder was homogenized with 0 1 M phosphate–citrate buffer, pH 7.2 (1 1, w/v), × 3 for 1 min, then extracted for 30 min with stirring The slurry was passed through two layers of cheesecloth and the resulting filtrate centrifuged at 35000 *g* for 30 min The supernatant was fractionated by $(NH_4)_2SO_4$ pptn between 20 and 55% satn at 0° The pellet which sedimented at 15000 *g* for 10 min was resuspended in 14 ml imidazole–HCl buffer (25 mM, pH 7) The soln was dialysed for 24 hr against 1 l of 25 mM imidazole–HCl buffer, pH 7, with three changes of buffer Insoluble material remaining after dialysis was removed by centrifugation.

Chromatofocusing was carried out at 4° on a column of Polybuffer exchanger 94 (1,2 × 60 cm, Pharmacia) equilibrated with 25 mM imidazole–HCl buffer, pH 7, using a flow rate of 22 ml/hr After application of the sample (247 mg protein in 13 ml) the column was eluted with Polybuffer 74 (1 10 dilution, pH 4, Pharmacia) and 3 ml fractions were collected Fractions corresponding to each activity peak were pooled separately and stored at −20° They were concd by TCA pptn before SDS-PAGE or by centrifugation under vacuum before isoelectric focusing and pore gradient electrophoresis

Electrophoretic methods and lipoxygenase detection SDS-PAGE was carried out according to ref. [34] in 15% acrylamide gels Isoelectric focusing was performed according to the conditions described earlier [35]. Polyacrylamide gradient gels (PAA 4/30 from Pharmacia) were used as recommended by the manufacturer except for the omission of EDTA from the electrophoresis buffer Lipoxygenase activity was detected by a modification of the Verhue and Francke method [13] The modification involved a 30 min incubation of the gel in air-saturated substrate soln (25 mM linoleic acid containing 0.7% Tween 20 in phosphate–citrate buffer, pH 6 35) with agitation. Then the dye was added (0 025% 3,3'-dimethoxybenzidine hydrochloride and 2 5% EtOH) The orange hydroperoxide bands which developed slowly (*ca* 48 hr) were stable to storage

Substrate emulsion. Linoleic acid stock soln was prepared using a modification of the method of ref [36] The substrate emulsion containing 35 6 mM linoleic acid and 1 vol % Tween 20 was stored under N_2 at 4° in the dark for not longer than 3 days

Assay of lipoxygenase activity Activity was determined polarographically at 25° with a Clark O_2 electrode Concns of linoleic acid were as indicated in the figure legends Incubation mixtures contained linoleic acid in 0 1 M phosphate–citrate buffer, pH 6 35, and were air saturated at 25° The reaction was initiated by addition of the enzyme Enzyme activities were expressed in μmol O_2 uptake/min Lipoxygenase activity was also measured by spectrophotometry at 234 nm under the same conditions A unit of lipoxygenase activity was defined as an increase in absorbance at 234 nm of 1 unit/min

Lipoxygenase activity in slices of gel was measured by polarography After electrophoresis, 5 mm segments of gel were immersed and crushed in 0 2 ml of phosphate–citrate buffer, pH 6 35, and this mixture was added to 1 33 mM linoleic acid, pH 6 35, in the reaction vessel

Other methods The chlorophyll *a* soln was prepared by the method of ref [24] Protein concentrations were determined by a Coomassie Brilliant Blue G250 binding assay [37] Oxidized linoleic acid soln was obtained after 8 days at +4° Hydroperoxide concentrations were determined by measuring A_{234} nm with 1 mM linoleic acid soln and using $\varepsilon_{234} = 25\,000$ [38]. Anaerobic conditions were obtained by permanent saturation of the reaction mixture with N_2 Samples were directly taken by means of a circulating cuvette (Spectrophotometer Spectronic 2000 Bausch et Lomb) for measurement of A_{234}, A_{285} and A_{668} Aerobic conditions were obtained by air-saturating the solns before addition of the enzyme and the cuvette was closed

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