Planta (1990) 181:35–43

Planta
© Springer-Verlag 1990

Lipoxygenase heterogeneity in *Pisum sativum*

C. Domoney[1], **J.L. Firmin**[1], **C. Sidebottom**[2], **P.M. Ealing**[1], **A. Slabas**[2], **and R. Casey**[1]

[1] John Innes Institute, Colney Lane, Norwich, NR4 7UH, and
[2] Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, UK

Abstract. Antibodies raised against two pea (*Pisum sativum* L. cv. Birte) seed lipoxygenases have been used to analyze lipoxygenase heterogeneity in seeds and in other organs. At least seven different polypeptides were identified in vivo; five of these were identified as precursors synthesized in vitro. The developmental appearance of the seed polypeptides has been analyzed and 'early' and 'late' forms were identified. Limited N-terminal sequence data indicated further heterogeneity when compared with sequences predicted from cDNAs.

Key words: Lipoxygenase (mRNA, protein) –*Pisum* (lipoxygenase) – Seed development

Introduction

Lipoxygenases are commercially important enzymes that catalyze the oxygenation of fatty acids and other compounds and appear to be widespread throughout the animal and plant kingdoms. Although lipoxygenases are involved in important reactions in animals where the lipoxygenase-derived metabolites of arachidonic acid elicit hypersensitive and inflammatory responses, no clearly defined physiological role has been demonstrated for the plant enzymes. Plant lipoxygenases have been implicated in flavour and odour formation, plant cell senescence by way of the formation of fatty-acid hydroperoxides and-or jasmonic acid and may play a role in responses to pest attack and wounding (for review, see Hildebrand 1989).

Lipoxygenases have been isolated from many plants but have been most studied in soyabean seeds where they constitute 1–2% of the seed protein (Vernooy-Gerritsen et al. 1983). The plant isoenzymes, which are monomeric proteins of relative molecular mass (M_r) approx. 95000, have been classified into type-1 and type-2 classes that differ in heat stability, pH optimum and substrate preference (Galliard and Chan 1980). In soyabeans, the former class contains lipoxygenase-1 and the latter class lipoxygenase-2 and lipoxygenase-3; lipoxygenase-3 can be chromatographically separated into two fractions, 3A and 3B. The nomenclature used for the animal enzymes is more explicit through classification as 5-, 8-, 9-, 11-, 12- and 15-lipoxygenases, based on the site of insertion of molecular oxygen on the fatty acid, usually arachidonic acid. The linoleic-acid-derived products of plant lipoxygenases usually contain hydroperoxide groups at C-9 or C-13 but assignment of positional specificity is not simple (see Galliard and Chan 1980). A lipoxygenase was purified from potato tubers which catalyzed the synthesis of a leukotriene from arachidonic acid in a two-step reaction employing separate 5- and 8-lipoxygenation steps (Shimizu et al. 1984). A 5-lipoxygenase has also been isolated from wheat (Kühn et al. 1985) and tulips (Reddanna et al. 1988). Lipoxygenases have been isolated from peas and there are conflicting reports as to the number, and molecular weights, of the isoenzymes. One or two enzymes of M_r 67000, 72000, 78000, 98000 or 106000 have been isolated from pea seeds (Eriksson and Svensson 1970; Arens et al. 1973; Haydar et al. 1975; Spaapen et al. 1977); later work described two major enzymes of M_r 95000 in pea seeds corresponding to soyabean lipoxygenases-2 and -3, and two minor enzyme fractions, one of which corresponds to soyabean lipoxygenase-1 (Yoon and Klein 1979). All of these analyses are based on the isolation of peaks of enzyme activity from column-chromatographic separations; none has employed Laemmli (1970) sodium dodecyl sulfate (SDS) gel electrophoresis or immunological analyses. Another analysis of pea seedlings identified five active lipoxygenase fractions and the proportion of these varied in different tissues (Anstis and Friend 1974a, b).

Abbreviations: cDNA = complementary DNA; DAF = days after flowering; HPLC = high-performance liquid chromatography; Ig = immunoglobulin; kb = kilobase; M_r = relative molecular mass; PAGE = polyacrylamide gel electrophoresis; PVDF = polyvinylidene difluoride; SDS = sodium dodecyl sulphate; SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0

The three major soyabean seed lipoxygenases show extensive homology; the predicted amino-acid sequences of lipoxygenase-1 and -3 are 81 and 74% identical to lipoxygenase-2, respectively (Shibata et al. 1987, 1988; Yenofsky et al. 1988). Human 15- and 5-lipoxygenases, on the other hand, show only 39% identity but comparison of these and plant sequences show that significant homology is restricted to two areas that may be important to function (Sigal et al. 1988; Dixon et al. 1988; Matsumoto et al. 1988). We have previously identified a complementary DNA (cDNA) clone corresponding to one of the major pea-seed lipoxygenases (Casey et al. 1985) and the predicted amino-acid sequence of a near full-length version of this cDNA and that of a soyabean lipoxygenase-3 clone show 86% identity (Ealing and Casey 1988; Yenofsky et al. 1988).

In the hope of elucidating some of the functions of lipoxygenases in plants, we are studying the developmental regulation of lipoxygenase synthesis during the life-cycle of *Pisum* (pea). The present work presents a profile, using antibodies raised against two seed components, of pea lipoxygenases present in seeds and in other organs. We also present some N-terminal sequence data obtained for some of the isolated polypeptides and compare these with predicted sequences from cDNAs.

Material and methods

Plant material. Dried seed of the *Pisum sativum* cv. Birte or the genotypes BC1/7RR and BC1/7rr (Hedley et al. 1986) were used. Developing seed were harvested from second and third flowering nodes of greenhouse-grown plants of cv. Birte. Young leaves, flowers, stems and roots were harvested from greenhouse-grown plants of cv. Birte which had three to five flowering nodes; roots were taken from approximately three-week-old plants growing in a chickgrit:Perlite mixture.

Other materials. Amicon cones (CF 50A) were purchased from Amicon Corp., Danvers, Mass., USA. Protein markers for gels were purchased from Sigma, Poole, Dorset, UK. Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore, Harrow, Mddx., UK.

Protein preparation. Dry seed or acetone-extracted freeze-dried fresh seed of cv. Birte were ground to a fine powder and extracted in 10 volumes of 50 mM sodium-phosphate buffer, pH 6.8 for 5 h at 4° C. The extract was filtered through cheesecloth and cleared by centrifugation at 16000·*g* for 15 min. Protein which precipitated between 25% and 60% relative saturation with ammonium sulphate was recovered from the supernatant, dissolved in and dialyzed extensively against 50 mM sodium-phosphate buffer, pH 6.8 at 6° C.

Between 0.5 and 2 g protein were applied to a Sephadex G200 column (79 cm long, 5 cm i.d.) equilibrated at 6° C with 50 mM sodium-phosphate buffer, pH 6.8. Fractions (8 ml) were eluted in the same buffer from the column (flow rate 0.5 ml/min) at 6° C. Lipoxygenase polypeptides were recovered from active fractions by precipitation with 60%-relative-saturation ammonium sulphate and dissolved in 50 mM sodium-phosphate buffer, pH 6.8.

For fractionation by high-performance liquid chromatography (HPLC), portions of Sephadex-G200-fractionated material were dialyzed against 0.02 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-acetate, pH 8 (Ramadoss and Axelrod 1982). Portions (1 mg) of protein were fractionated at 10° C on a column (TSKgel DEAE-5PW; Anachem, Luton, Beds., UK; 75 mm long, 7.5 mm i.d.; flow rate 1 ml/min) with a linear gradient formed from a starting buffer of 0.02 M Tris-acetate, pH 8 and a final buffer of 0.02 M Tris-acetate containing 0.5 M sodium acetate, pH 8.25. Fractions corresponding to peaks were pooled and concentrated in Amicon cones.

Antibody preparation. For injection of rabbits, polypeptides were further purified by elution from 7.5% SDS polyacrylamide gels (PAGE). Gels were stained with Coomassie Brilliant Blue G, destained briefly and dried. Bands were excised from dried gels, rehydrated and the polypeptides extracted from the gel slices as previously described (Domoney and Casey 1983). Rabbits were injected at two-weekly intervals with approx. 150 μg protein. Immunoglobulin Gs (IgGs) were precipitated from sera and dissolved in and dialyzed against phosphate-buffered saline (PBS). Anti-lipoxygenase IgG was obtained by affinity chromatography on CNBr-Sepharose 4B columns to which protein highly enriched in lipoxygenase polypeptides had been covalently linked. Specific IgGs were eluted from affinity columns with low-pH buffer or using 3 M potassium isothiocyanate (KSCN) (Kerckaert et al. 1977; Casey 1979). Lipoxygenase polypeptides were affinity purified on CNBr-Sepharose 4B columns to which affinity-purified anti-lipoxygenase IgG had been covalently linked.

Protein analyses. Pea proteins were quantified by the dye-binding method of Bradford (1976), using bovine serum albumin as standard, and analyzed by PAGE [10% (w/v) gels]. Protein samples were prepared for electrophoresis by boiling in Laemmli (1970) sample buffer for 5 min. Developing seed and samples of other tissue were first freeze-dried and then extracted by boiling in sample buffer. Immunoblotting was performed as previously described (Casey et al. 1985) using an overnight incubation of filters with a 1 in 2500 dilution of rabbit anti-pea lipoxygenase IgG (1 mg/ml) or a 1 in 800 dilution of goat anti-soyabean lipoxygenase IgG (1 mg/ml; a gift from Dr N. Nielsen, Purdue University, West Lafayette, Ind., USA).

Lipoxygenase activity was measured using a polarographic method and linoleic acid as substrate.

Analysis of RNA. The preparation and translation of RNA and immunoprecipitation of translation products were as previously described (Domoney and Casey 1987). The RNAs were analyzed by dot-blot and 'Northern' hybridizations, as described (Domoney and Casey 1983, 1987) except that hybridization conditions were as described by Domoney and Casey (1985). The isolation of a cDNA clone corresponding to one of the major seed lipoxygenases has been described (Casey et al. 1985) and pPE1036 represents a near full-length version of this (Ealing and Casey 1988). The cDNA clone pPE923 represents the cDNA class which apparently corresponds to the other major pea seed lipoxygenase polypeptide (Casey et al. 1985; Ealing and Casey 1988).

Analysis of N-terminal amino-acid sequences. Lipoxygenase polypeptides were resolved by PAGE [7.5% (w/v) gels]. Preparation of samples and electrophoresis conditions were such as to minimize N-terminal blocking, following the recommendation of Applied Biosystems (Foster City, Cal., USA; Yuen et al. 1986). Protein was electroblotted onto PVDF membranes in 10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 10% methanol, pH 11 for 1 h at 0.5 A and the bands visualized with Coomassie Brilliant Blue R as described by Matsudaira (1987). Bands were cut out and stored at −20° C. It was estimated, from the intensity of Coomassie blue staining, that approx. 50–100 pmol of each polypeptide was loaded onto the initial gel. Sequential Edman degradation was performed on an Applied Biosystems (ABI) gas-phase sequencer, model 470. Phenylthiohydantoin (PTH) derivatives were identified and quantified using an ABI 120 PTH analyser, following the manufacturer's recommended procedures. Initial sequencer yields for samples A1, C1 and B were 6, 11 and 20 pmol, respectively.

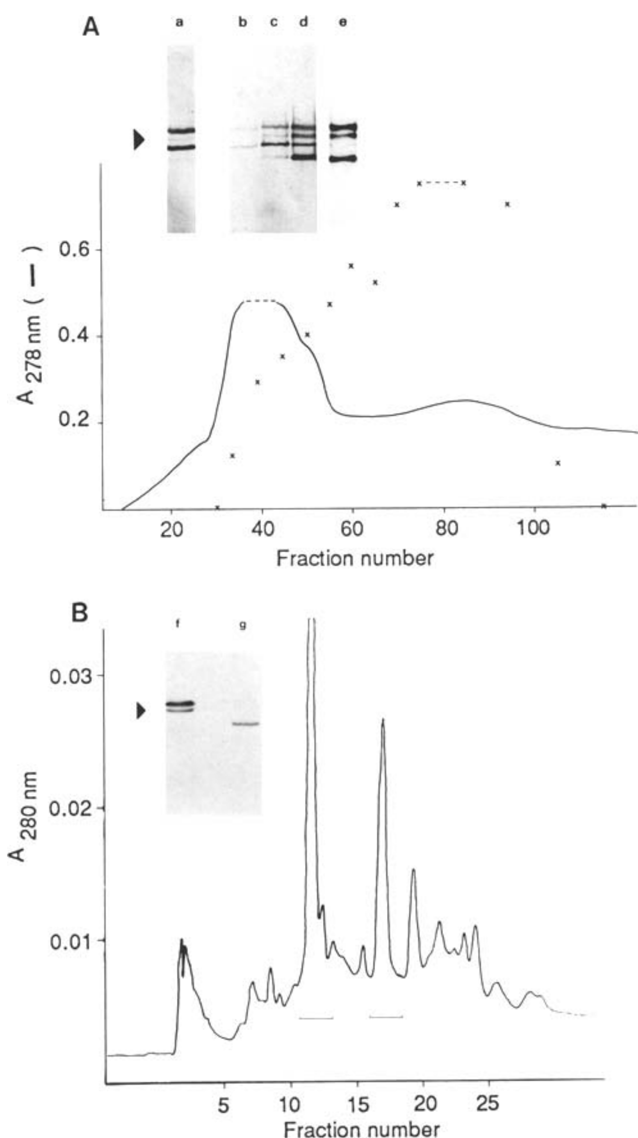


Fig. 1 A, B. Separation of pea (cv. Birte) seed lipoxygenase fractions by column chromatography. The *solid line* shows absorbance at 278 (**A**) or 280 (**B**) nm. *Broken lines* indicate off-scale measurements. *Arrowheads* indicate the position of phosphorylase B (M_r 97400). **A** Sephadex-G200 separation of ammonium-sulphate-fractionated protein. Lipoxygenase activity (arbitrary units) of selected fractions is indicated (X). *Inset*: Western-blot analysis of the starting material (*track a*) or of pooled fractions through the peak of lipoxygenase activity (*tracks b–e*). **B** Fractionation by HPLC anion-exchange of the protein fraction shown in **A**, *track e*. The linear gradient was 0–100% of the final buffer in 40 min (i.e. fraction 40) (not shown). *Inset*: The protein in the peaks indicated ⎵ was analyzed by PAGE and stained with Coomassie Brilliant Blue G (1st and 2nd peaks shown in *tracks f* and *g*, respectively)

Results

Purification of lipoxygenases. The profile of lipoxygenase activity in relation to absorbance at 278 nm of pea (cv. Birte) seed proteins separated by Sephadex-G200 chromatography (Fig. 1 A) is consistent with a relatively low molecular mass (a monomeric protein of M_r approx. 100000). Samples of starting material (a) and samples through the peak of lipoxygenase activity (b–e) were analyzed by PAGE, blotting and incubation with anti-soybean lipoxygenase IgG. Two major polypeptides evident in track a correspond to those previously identified in crude extracts of pea meal (Casey et al. 1985). Two additional immunoreactive polypeptides, evident in tracks c and d, are considerably enriched in e. Traces of these 'minor' polypeptides are evident in track a and also in crude pea-meal extracts (not shown). In an attempt to purify the relatively minor polypeptides, the protein fraction shown in track e was subjected to ion-exchange chromatography (HPLC column). Figure 1 B shows a typical separation; analysis of the two main peaks by PAGE showed a separation of the apparently smaller polypeptide (g) from the other two (f). It did not prove possible to separate easily the two polypeptides shown in track f. Therefore, these polypeptide preparations were subjected to preparative gel electrophoresis and an antibody was raised against the mixture of bands seen in track f and another against the polypeptide shown in track g. Antibody raised against the polypeptides shown in track f is called anti-A whereas that raised against the polypeptide shown in track g is called anti-B.

The specificities of the two antibody preparations compared with anti-soyabean lipoxygenase IgG on Western blots are shown in Fig. 2, tracks a–i. Reaction with anti-B (d–f) is very similar to that obtained with anti-soyabean lipoxygenase IgG (g–i) whereas anti-A (a–c) shows a more specific reaction toward some of the polypeptides in pea meal (a, d, g), ammonium-sulphate-fractionated protein (b, e, h) and the Sephadex-G200 fraction used for HPLC analysis (c, f, i). However, the specificities of the two pea antibodies on 'Western' blots were not related to their specificities when used as affinity columns to purify lipoxygenases. Figure 2, tracks k, m, n, shows the polypeptides obtained by affinity chromatography of the mixtures shown in tracks j and l on anti-B affinity columns. (In Fig. 2, j–n represent Coomassie-stained gel tracks.) When ammonium-sulphate-fractionated protein is used (j), one of the major lipoxygenase polypeptides is specifically purified (k); when the protein fraction used for HPLC analysis (l; also shown in immunoblotted tracks f and i) is used for anti-B affinity columns, specific enrichment is also achieved (m). However, an additional minor component is evident among the affinity-purified polypeptides (k, m) which is of an apparently lower M_r than those previously identified. This component could be enriched (n) by affinity chromatography of other protein fractions (not shown). Affinity chromatography on anti-A affinity columns showed no specific enrichment of any of the lipoxygenase-related polypeptides (data not shown) in contrast to the specificity of anti-A IgG on Western blots (a–c). Thus there are five lipoxygenase polypeptides identifiable in pea seed by a combination of column and affinity chromatography and Western blotting. The results presented are those obtained from dry seed but similar results were obtained by fractionation of protein prepared from developing seed; in addition, results obtained from

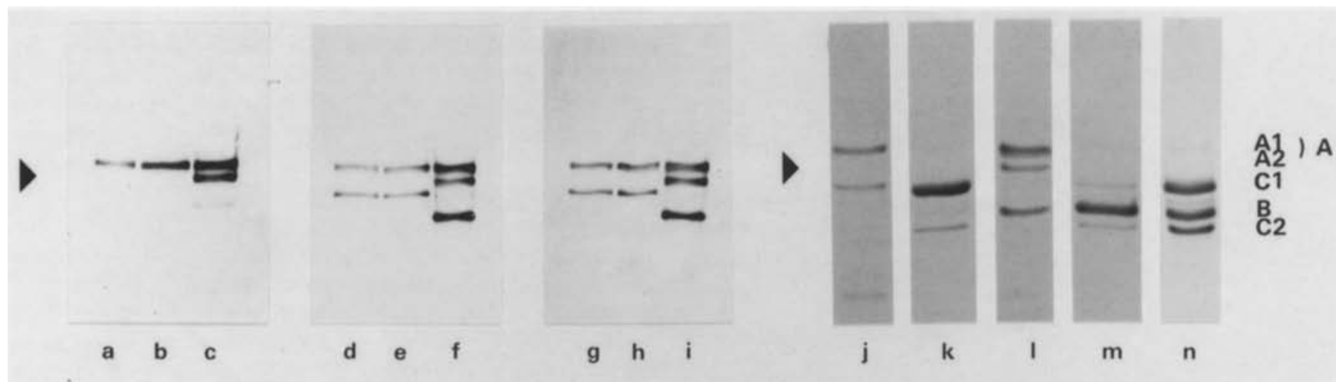


Fig. 2. An analysis by PAGE and Western blotting (*tracks a–i*) or Coomassie staining (*tracks j–n*) of pea (cv. Birte) seed lipoxygenase polypeptides related to the antibody preparations. The blots show reaction of pea meal (*a, d, g*), ammonium-sulphate-fractionated protein (*b, e, h*) and Sephadex-G200-fractionated protein (*c, f, i;* equivalent to track e in Fig. 1A) to anti-A (*a–c*), anti-B (*d–f*) and anti-soyabean (*g–i*) IgGs. Affinity-purified lipoxygenases (*k, m, n*) were obtained by chromatography of ammonium-sulphate-fractionated protein (*j*) or Sephadex-G200-fractionated protein (*l*) on anti-B affinity columns. The protein shown in *k* was derived from *j* and that shown in *m* and *n* was derived from fractions similar to *l*. The lipoxygenase polypeptides are labelled A1, A2, C1, B, C2 in order of increasing mobility. *Arrowheads* indicate the position of the marker of M_r 97400

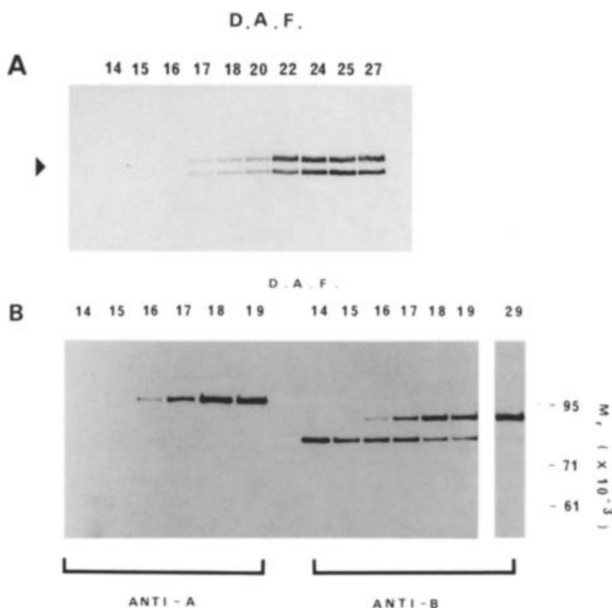


Fig. 3A, B. An analysis of embryos harvested at 14–29 DAF by Western blotting using anti-B IgG (**A**) or by RNA translation and immunoprecipitation using anti-A or anti-B IgGs as indicated (**B**). In **A**, the *arrowhead* indicates the position of the marker of M_r 97400. In **B**, the positions of three labelled markers are indicated

two other genotypes (BC1/7RR, BC1/7rr) were very similar to those obtained from cv. Birte (data not shown).

Appearance of lipoxygenase during seed development. Figure 3A shows an analysis by PAGE and blotting (using anti-B IgG) of developing seed (whole seed meal) harvested 14–27 days after flowering (DAF). It is clear that the two major lipoxygenase polypeptides are first detected at precisely the same stage (17 DAF).

When RNA was isolated from developing seed, translated and the products immunoprecipitated with anti-A IgG, one polypeptide was evident from 16 DAF onwards (Fig. 3B). This polypeptide corresponds in size to the upper of the two polypeptides evident in the blot shown in Fig. 3A. When translation products were immunoprecipitated with anti-B IgG, a polypeptide corresponding in size to the lower of the two polypeptides shown in Fig. 3A was also evident from 16 DAF onwards. (The specificities of the antibodies for the precursor polypeptides thus differ from the specificities observed for SDS-denatured or native proteins.) However, an additional precursor polypeptide was immunoprecipitated with anti-B IgG; it represents a decreasing proportion of the anti-B immunoprecipitable polypeptides as development proceeds and is present only as a very minor component later in development (29 DAF shown, Fig. 3B). These immunoprecipitation patterns were also observed for developing embryos of the genotype BC1/7RR and indicate that a minor lipoxygenase polypeptide is the predominant form synthesized early in seed development before the major forms appear. The anti-B immunoprecipitable polypeptide present at 14 and 15 DAF (Fig. 3B) was detected in translation products of RNA from the earliest embryos examined (1 mg fresh weight; not shown). The relative mobility of this 'early' polypeptide is coincident with the affinity-purified C2 polypeptide (Fig. 2, k, m, n).

The hybridization of inserts from cDNAs representing the two major seed lipoxygenase polypeptides (Ealing and Casey 1988) to RNA isolated from embryos throughout development shows that RNA corresponding to these clones is detected only after 15 DAF (Fig. 4A). There was no difference in the time of appearance nor in the sizes (approx. 3 kb) of the mRNAs detected with the two cDNAs pPE1036 (Fig. 4A, i) or pPE923 (Fig. 4A, ii) under conditions where cross-hybridization of the two probes was at a minimum. Hybridization of either probe at lower stringencies did not reveal any hybridization to RNA before 16 DAF (Fig. 4A, iii); hence, neither probe has significant homology with RNA representing the 'early' lipoxygenase

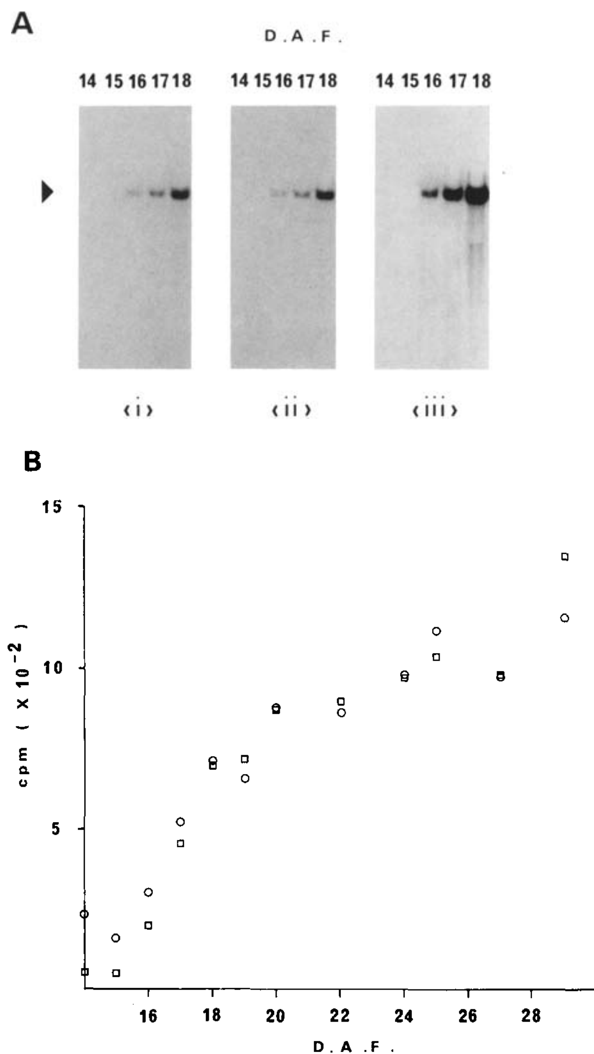


Fig. 4A, B. An analysis of lipoxygenase mRNAs during pea seed development. Embryos were harvested at 14–29 DAF and used for Northern blotting (**A**) or dot-blotting (**B**). **A** Northern blots (approx. 3 μg total RNA per sample) were hybridized to inserts from the plasmids pPE923 (*i*), pPE 1036 (*ii*), (*iii*). Filters were washed to a stringency of 0.1 × SSC, 0.1% SDS at 65° C (*i*), (*ii*) or 2 × SSC, 0.1% SDS at 50° C (*iii*). *Arrowhead* indicates position of a DNA marker of approx. 3 kb. A minor band evident in (*iii*) is approx 2 kb. **B** Dot-blots were hybridized to inserts from the plasmids pPE923 (○) or pPE1036 (□) and radioactivity was measured by scintillation counting

polypeptide evident among translation products before 16 DAF (Fig. 3B).

Quantitation of lipoxygenase RNAs homologous to pPE1036 and pPE923 by dot-blot hybridization shows that the amounts of both RNAs increase throughout seed development (Fig. 4B); this is in contrast to the situation observed for storage-protein mRNAs which show clear maxima at distinct stages of development and thereafter decrease in amount (Chandler et al. 1984; Domoney and Casey 1987). The amount of hybridization to both probes was very similar; in addition hybridization under lower stringency to either probe resulted in approximately twice the amount of hybridization (data not shown). These observations indicate that steady-state RNA levels corresponding to the two probes are very similar.

Lipoxygenase polypeptides in other organs. Analysis of extracts from flowers, stems and roots by PAGE and immunoblotting using anti-B IgG showed patterns of lipoxygenase polypeptides which differed from those observed for seed (Fig. 5A). There was no obvious reaction with leaf extracts (Fig. 5A, f). Analysis of mixtures of seed extracts or seed-protein fractions and extracts from flowers, stems or roots showed that the main stem and root polypeptides (Fig. 5A, c, d) differed in M_r from any of the seed polypeptides (Fig. 5A, a, e). The main polypeptide observed in flower extracts co-migrated with a minor seed polypeptide (Fig. 5A, compare b and e with k). A minor root polypeptide (Fig. 5A, d) appeared to co-migrate with a minor seed polypeptide (C2) observed in affinity-purified fractions (compare with Fig. 2, k, m, n). The patterns observed for flower, stem, root and leaf extracts were the same whether anti-A (not shown) or anti-B IgGs were used.

Translation of RNA isolated from these other organs and immunoprecipitation with anti-B IgG showed a predominant immunoprecipitable polypeptide and, except in the case of leaf RNA, other more minor polypeptides (Fig. 5B). For comparison, the products of RNA from developing seed (at 17 DAF) which were immunoprecipitated with both anti-A and anti-B IgGs are included (Fig. 5B, Sd). This comparison shows that the predominant immunoprecipitable product from other organs comigrates with the 'early' lipoxygenase of seeds (compare with Fig. 3B).

The more minor immunoprecipitable products observed for flowers, stems and roots are of similar mobility to those observed in Western blots (Fig. 5A, b, c, d) except for an additional polypeptide observed in translation products from flower RNA (Fig. 5B). The relative abundance of the immunoprecipitable products shown in Fig. 5B for flower, root and stem RNAs is not reflected in their relative abundance when total translation products are examined (data not shown); in the latter case, all the lipoxygenase-related polypeptides are of approximately equal intensity. It appears, therefore, that their relative abundance on immunoprecipitation is caused by differential affinity of the antibody for the translation products. The apparently minor immunoprecipitated products evident for flower, root and stem RNAs are immunoprecipitated to the same extent by anti-A IgG (data not shown), indicating that they show an 'intermediate' reaction to the two antibodies. This is in contrast to the situation for seed translation products which are immunoprecipitated by only one of the two antibodies (Fig. 3B); in addition, in the case of seed RNA, the degree of precipitation of a product was related to its intensity among total products (not shown).

Figure 5C shows a Northern-blot analysis of RNA from the different organs using the seed cDNA clone, pPE1036. Hybridization to flower, root and stem RNA

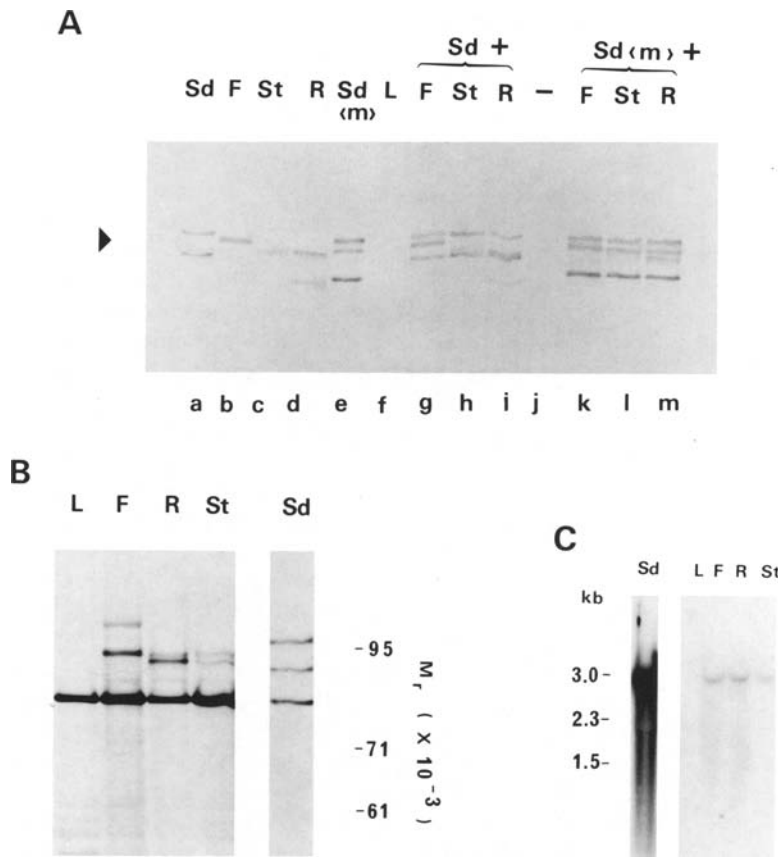


Fig. 5A–C. An analysis of lipoxygenase polypeptides and mRNAs from different pea organs. Samples were analyzed by PAGE and Western blotting (**A**), RNA translation and immunoprecipitation (**B**) or Northern blots (**C**) of RNA or protein from seed/embryo (*Sd*), leaf (*L*), flower (*F*), stem (*St*) and root (*R*) tissues. The Sephadex-G200-fractionated seed protein enriched in minor polypeptides is shown in (**A**) as *Sd<m>*. **A** Western blot showing an analysis of flower, stem and root extracts to which crude seed meal extract (Sd) or Sephadex-G200-fractionated protein (*Sd<m>*) had been added (*Sd+F* etc). Blots were reacted with anti-B IgG. The *arrowhead* indicates the position of the marker of M_r 97400. **B** The immunoprecipitated products were obtained using anti-B IgG (*L, F, R, St*) or a mixture of anti-A and anti-B IgGs (*Sd*). **C** The 'Northern' blot was hybridized to the insert from the plasmid pPE1036 and washed to a stringency of 2×SSC, 0.1% SDS at 50° C. The positions of DNA markers are indicated

was evident only on long exposures of comparable amounts of seed RNA. In addition, no hybridization to leaf RNA could be detected, indicating that the probe used does not hybridize to RNA representing the most abundant immunoprecipitable product. This was also the case on Northern analysis of early developing seed (Fig. 4A) and is further evidence that the 'early' seed lipoxygenase translation product and the leaf translation product are the same.

Lipoxygenase N-terminal analyses. Figure 6A shows an electroblot of lipoxygenase samples onto PVDF membranes; the bands, which are clearly resolved, were excised and used for sequencing. Figure 6B shows a comparison of the N-termini of three of the polypeptides compared to the N-terminal amino-acid sequences deduced from the sequences of pPE1036 (Ealing and Casey 1988) and pPE923. The deduced sequence of pPE1036 represents a full-length protein sequence. It is notable that, although all of these sequences are derived from cv. Birte, no two are identical. The sequence of C1 matches most closely the deduced sequence of pPE923, but extends N-terminally to the deduced sequence. Comparison of the N-terminal amino acids of C1 and A1 relative to the deduced sequence of pPE1036 indicates that removal of amino acids other than the initiator methionine may occur post-translationally during lipoxygenase synthesis. On the other hand the N-terminal amino acids F, S and G of the deduced sequence of pPE1036 are absent from the deduced sequence of a soyabean lipoxygenase-3 clone (Yenofsky et al. 1988). The protein corresponding to this last sequence is N-terminally blocked and it may be significant that the blocked amino acid is a (deduced) leucine whereas the pea sequences with N-terminal valine (C1) or glycine (A1) are not blocked.

The sequence of pea lipoxygenase B shows that it is truncated relative to the other sequences, but it is extremely similar to the deduced sequence of pPE923 (Fig. 6B). Some heterogeneity was detected at two positions of this sequence, in agreement with analysis of this polypeptide by two-dimensional gel electrophoresis (O'Farrell 1975) which showed it to be composed of two spots (data not shown). Since a polypeptide corresponding in size to pea lipoxygenase B was not evident among translation products, it is possible that B arises post-translationally by removal of a stretch of N-terminal amino acids. Since this lipoxygenase polypeptide could be detected on fractionation of extracts of developing seed, it is unlikely that the modification takes place during desiccation of the seed. It is possible that it occurs artefactually during preparation of protein; this seems unlikely as a trace of B can be detected in seed meal immediately extracted in PAGE sample buffer and, in addition, rapid protein preparations did not appear to affect the relative amount of B recovered.

The C2 polypeptide enriched in affinity-purified lipoxygenases (Fig. 2, n) which corresponds to the "ear-

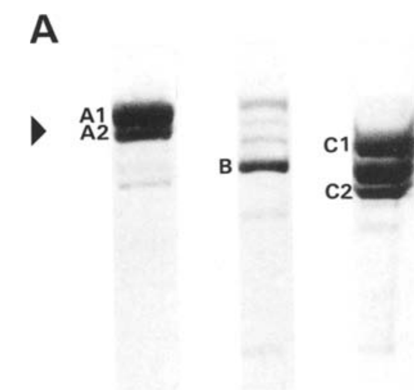
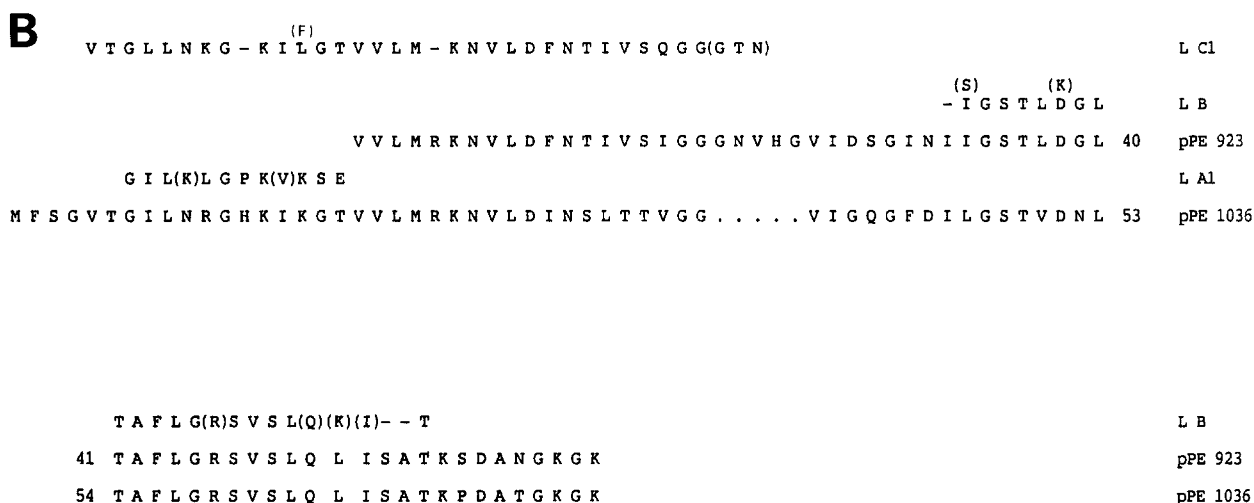


Fig. 6A, B. N-terminal sequence analysis of lipoxygenase polypeptides. **A** The separated polypeptides on a stained PVDF membrane used for sequencing. Samples enriched for A1, A2 and B were obtained by HPLC fractionation. The sample enriched for C1 and C2 was obtained by affinity chromatography on anti-B columns. The *arrowhead* indicates the position of the marker of M_r 97400. **B** A comparison of the N-terminal sequences obtained for the lipoxygenase A1, B and C1 polypeptides compared with the sequences predicted from the cDNA clones pPE923 and pPE1036. Bracketed residues within the determined sequences indicate uncertainties; bracketed residues over the sequences indicate heterogeneity of the determined sequence; a dash indicates that a residue could not be identified. Gaps (*dots*) are introduced in the predicted sequence of pPE1036 to facilitate alignment



ly" seed translation product (Fig. 3B) was N-terminally blocked, as was the minor component of lipoxygenase A (A2; see Fig. 2C).

Discussion

Antibodies raised against two seed lipoxygenase fractions were used to examine lipoxygenase heterogeneity in *Pisum*. The two antibody preparations were found to react differentially with the various lipoxygenase polypeptides and the reactions observed varied according to whether SDS-denatured protein, native protein or primary translation products were being examined.

The results presented here show that, in addition to two major seed lipoxygenases (Casey et al. 1985; North et al. 1989), a number of minor lipoxygenases are detectable in pea seed extracts. A combination of Western-blot analysis, immunoaffinity chromatography in conjunction with other protein purifications and immunoprecipitation of cell-free translation products was used to analyze the complexity of the patterns observed. Of five different seed lipoxygenase polypeptides identified in vivo, three (A1, C1, C2) were evident among immunoprecipitated translation products. The remaining two in-vivo polypeptides may thus arise by post-translational modifications of some of the primary translation products. One (lipoxygenase B) was shown by N-terminal sequence analysis to be truncated relative to other sequences; microheterogeneity of B indicated that at least two different polypeptides were present. The A2 polypeptide was N-terminally blocked and hence its origin cannot be speculated upon. It is interesting to note, however, that the A2 seed polypeptide appeared to co-migrate with the main immunoreactive polypeptide from flowers; in the latter case, this polypeptide was detected as a primary translation product. Further information will be required to establish whether these two polypeptides represent products of the same gene. Extracts of stems and roots showed reaction to a pair of polypeptides of very similar M_r which were distinct from the five seed polypeptides. Thus a minimum of seven different polypeptides were identified by Western-blot analysis.

The major seed lipoxygenases were first detected at the protein level at 17 DAF and at the RNA level at 16 DAF. This onset of synthesis of the major lipoxygenases is relatively late compared with the onset of seed storage-protein synthesis; e.g. both vicilin and legumin translation products are easily detected at 14 DAF (Domoney and Casey 1987). Translatable RNA corresponding to one of the minor seed polypeptides observed in vivo (C2) was detected in the earliest developing seed examined (1 mg, where it represented a predominant

translation product; data not shown) and was the only lipoxygenase detected in leaves. C2 was detected as a translation product in all other organs examined but only in roots could it be detected on Western analysis of crude extracts, indicating that it may be relatively more abundant in roots. This latter observation and the apparent ubiquity of this lipoxygenase indicates that it plays an important role in cell development and may be closely related to processes of cell division or cytokinesis; its relative abundance in 1 mg embryos, where close to 100% of the cell population are potentially dividing cells (Ambrose et al. 1987), supports this notion. On the other hand, it represents a very minor component of immunoprecipitable polypeptides at late stages of seed development where there is little or no cell division taking place (Ambrose et al. 1987). The C2 polypeptide may thus fulfil a more general 'housekeeping' function in contrast to other lipoxygenase polypeptides. The results of Northern hybridizations indicate that RNA corresponding to C2 does not hybridize to cDNA clones representing the major seed lipoxygenases; this indicates limited homology between C2 and the clones pPE1036 and pPE923 and may represent a situation analogous to two classes of pea legumin gene which share limited homology at the nucleic acid level (Domoney and Casey 1985; Gatehouse et al. 1988). It is unlikely that C2 corresponds to lipoxygenase-1 which is a very minor component of pea lipoxygenase (Yoon and Klein 1979), as extensive homology exists between lipoxygenases -1, -2 and -3 from soyabean (Shibata et al. 1987, 1988; Yenofsky et al. 1988); C2 may correspond to a second minor component identified in pea lipoxygenase by Yoon and Klein (1979).

Isolation of cDNAs and-or genes corresponding to C2 may allow further deductions to be made regarding differences in expression. The results presented here show that the timing of synthesis, and location, of this polypeptide differ dramatically from those of all the other lipoxygenases identified.

This work was supported by the Agricultural and Food Research Council via a grant-in-aid to the John Innes Institute. We acknowledge financial support from the Commission of the European Communities Biotechnology Action Programme; grant No. 0063-UK.

References

Ambrose, M.J., Wang, T.L., Cook, S.K., Hedley, C.L. (1987) An analysis of seed development in *Pisum sativum*. IV. Cotyledon cell populations in vivo and in vitro. J. Exp. Bot. **38**, 1909–1920

Anstis, P.J.P., Friend, J. (1974a) The effect of light on lipoxygenase activity in dwarf pea seedlings. Phytochemistry **13**, 2709–2712

Anstis, P.J.P., Friend, J. (1974b) The isoenzyme distribution of etiolated pea seedling lipoxygenase. Planta **115**, 329–335

Arens, D., Seilmeier, W., Weber, F., Kloos, G., Grosch, W. (1973) Purification and properties of a carotene co-oxidizing lipoxygenase from peas. Biochim. Biophys. Acta **327**, 295–305

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

Casey, R. (1979) Immunoaffinity chromatography as a means of purifying legumin from *Pisum* (pea) seeds. Biochem. J. **177**, 509–520

Casey, R., Domoney, C., Nielsen, N.C. (1985) Isolation of a cDNA clone for pea (*Pisum sativum*) seed lipoxygenase. Biochem. J. **232**, 79–85

Chandler, P.M., Spencer, D., Randall, P.J., Higgins, T.J.V. (1984) Influence of sulfur nutrition on developmental patterns of some major pea seed proteins and their mRNAs. Plant Physiol. **75**, 651–657

Domoney, C., Casey, R. (1983) Cloning and characterization of complementary DNA for convicilin, a major seed storage protein in *Pisum sativum* L. Planta **159**, 446–453

Domoney, C., Casey, R. (1985) Measurement of gene number for seed storage proteins in *Pisum*. Nucleic Acids Res. **13**, 687–699

Domoney, C., Casey, R. (1987) Changes in legumin messenger RNAs throughout seed development in *Pisum sativum* L. Planta **170**, 562–566

Dixon, R.A.F., Jones, R.E., Diehl, R.E., Bennett, C.D., Kargman, S., Rouzer, C.A. (1988) Cloning of the cDNA for human 5-lipoxygenase. Proc. Natl. Acad. Sci. USA **85**, 416–420

Ealing, P.M., Casey, R. (1988) The complete amino acid sequence of a pea (*Pisum sativum*) seed lipoxygenase predicted from a near full-length cDNA. Biochem. J. **253**, 915–918

Eriksson, C.E., Svensson, S.G. (1970) Lipoxygenase from peas, purification and properties of the enzyme. Biochim. Biophys. Acta **198**, 449–459

Galliard, T., Chan, H.W.-S. (1980) Lipoxygenases. In: The biochemistry of plants, vol. 4, pp. 131–161, Stumpf, P.K., ed. Academic Press, New York

Gatehouse, J.A., Bown, D., Gilroy, J., Levasseur, M., Castleton, J., Ellis, T.H.N. (1988) Two genes encoding 'minor' legumin polypeptides in pea (*Pisum sativum* L.). Characterization and complete sequence of the *LegJ* gene. Biochem. J. **250**, 15–24

Haydar, M., Steele, L., Hadziyev, D. (1975) Oxidation of pea lipids by pea seed lipoxygenase. J. Food Sci. **40**, 808–814

Hedley, C.L., Smith, C.M., Ambrose, M.J., Cook, S.K., Wang, T.L. (1986) An analysis of seed development in *Pisum sativum*. II. The effect of the *r*-locus on the growth and development of the seed. Ann. Bot. **58**, 371–379

Hildebrand, D.F. (1989) Lipoxygenases. Physiol. Plant. **76**, 249–253

Kerckaert, J.-P., Bayard, B., Debray, H., Sautière, P., Biserte, G. (1977) Rat alpha-fetoprotein heterogeneity. Comparative chemical study of the two electrophoretic variants and their *Ricinus* lectin-binding properties. Biochim. Biophys. Acta **493**, 293–303

Kühn, H., Heydeck, D., Wiesner, R., Schewe, T. (1985) The positional specificity of wheat lipoxygenase; the carboxylic group as signal for the recognition of the site of the hydrogen removal. Biochim. Biophys. Acta **830**, 25–29

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

Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**, 10035–10038

Matsumoto, T., Funk, C.D., Rådmark, O., Höög, J.-O., Jörnvall, H., Samuelsson, B. (1988) Molecular cloning and amino acid sequence of human 5-lipoxygenase. Proc. Natl. Acad. Sci. USA **85**, 26–30

North, H., Casey, R., Domoney, C. (1989) Inheritance and mapping of seed lipoxygenase polypeptides in *Pisum*. Theor. Appl. Genet. **77**, 805–808

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**, 4007–4021

Ramadoss, C.S., Axelrod, B. (1982) High-performance liquid chromatographic separation of lipoxygenase isozymes in crude soybean extracts. Anal. Biochem. **127**, 25–31

Reddanna, P., Whelan, J., Reddy, P.S., Reddy, C.C. (1988) Isolation and characterization of 5-lipoxygenase from tulip bulbs. Biochem. Biophys. Res. Comm. **157**, 1348–1351

Shibata, D., Steczko, J., Dixon, J.E., Hermodson, M., Yazdanpar-

ast, R., Axelrod, B. (1987) Primary structure of soybean lipoxygenase-1. J. Biol. Chem. **262**, 10080–10085

Shibata, D., Steczko, J., Dixon, J.E., Andrews, P.C., Hermodson, M., Axelrod, B. (1988) Primary structure of soybean lipoxygenase L-2. J. Biol. Chem. **263**, 6816–6821

Shimizu, T., Rådmark, O., Samuelsson, B. (1984) Enzyme with dual lipoxygenase activities catalyzes leukotriene A4 synthesis from arachidonic acid. Proc. Natl. Acad. Sci. USA **81**, 689–693

Sigal, E., Craik, C.S., Highland, E., Grunberger, D., Costello, L.L., Dixon, R.A.F., Nadel, J.A. (1988) Molecular cloning and primary structure of human 15-lipoxygenase. Biochem. Biophys. Res. Comm. **157**, 457–464

Spaapen, L.J.M., Vliegenthart, J.F.G., Boldingh, J. (1977) On the properties of a pea lipoxygenase. Biochim. Biophys. Acta. **488**, 517–520

Vernooy-Gerritsen, M., Bos, A.L.M., Veldink, G.A., Vliegenthart, J.F.G. (1983) Localization of lipoxygenases-1 and -2 in germinating soybean seeds by an indirect immunofluorescence technique. Plant Physiol. **73**, 262–267

Yenofsky, R.L., Fine, M., Liu, C. (1988) Isolation and characterization of a soybean (*Glycine max*) lipoxygenase-3 gene. Mol. Gen. Genet. **211**, 215–222

Yoon, S., Klein, B.P. (1979) Some properties of pea lipoxygenase isoenzymes. J. Agric. Food Chem. **27**, 955–962

Yuen, S., Hunkapiller, M.W., Wilson, K.J., Yuan, P.M. (1986) SDS-PAGE electroblotting. Applied Biosystems User Bulletin, Issue no. 25

Received 24 August; accepted 28 September 1989