High-Performance Liquid Chromatographic Analysis of the Products of Linoleic Acid Oxidation Catalyzed by Pea (*Pisum sativum*) Seed Lipoxygenases

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An HPLC method is discussed for the analysis of the products formed by the pea (*Pisum sativum*) lipoxygenase catalyzed oxidation of linoleic acid. The results demonstrate the feasibility of analyzing all of the hydroperoxides, hydroxides, and keto fatty acids in a single chromatographic step and show that it will be possible to analyze the product profile from the lipoxygenase activity contained in a portion of a seed, which will permit the remainder of the seed to be grown on for subsequent generations. The chemical structures of the products have been identified by HPLC analysis and GC–MS.

Keywords: *Lipoxygenase; hydroperoxides; chromatography*

INTRODUCTION

Lipoxygenases (LOX) are well-known for their ability to catalyze the hydroperoxidation of polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene structure. The primary reaction products with linoleic acid as substrate are some, or all, depending on the reaction conditions and the source of the enzyme (Whitaker, 1991; Gardner, 1991), of the following: 13-hydroperoxy-(9*Z*,11*E*)-octadeca-9,11-dienoic acid (13ZE-HPODE), 13-hydroperoxy-(9*E*,11*E*)-octadeca-9,11-dienoic acid (13EE-HPODE), 9-hydroperoxy-(10*E*,12*Z*)-octadeca-10,12-dienoic acid (9EZ-HPODE) and 9-hydroperoxy-(10*E*,12*E*)-octadeca-10,12-dienoic acid (9EE-HPODE). These HPODEs can be readily reduced either by LOX or by contaminating or associated proteins (Vliegenthart and Veldink, 1982) to the corresponding hydroxy fatty acids; in the case of the above, the 13ZE, 13EE, 9EZ, and 9EE hydroxyoctadecadienoic acids (HODEs) would be formed. In addition, carbonyl compounds have been detected under various reaction conditions with either soybean (Axelrod et al., 1981; Reynolds and Klein, 1982; Veldink and Vliegenthart, 1984; Hildebrand et al., 1990) or pea (Reynolds and Klein, 1982; Yoon and Klein, 1979; Kuhn et al., 1987) LOX.

Although hydroxy and keto fatty acids are secondary products, they could be formed simultaneously with the onset of the primary oxidation of the substrate; their separation from the hydroperoxides is therefore important for the assay of the primary and secondary reaction products. There are reports of the separation by HPLC of hydroxy fatty acids (Beneytout et al., 1989; Nikolaev et al., 1990; Gardner et al., 1991) and their methyl esters (Hughes et al., 1983) and of the partial separation of the fatty acid hydroperoxides (Yamamoto et al., 1980; Ohta et al., 1991); Teng and Smith (1985) have also reported the separation of the hydroperoxides and hydroxides. Several authors have reported the formation of keto fatty acids, but only Kuhn et al. (1991) have described their separation by HPLC and determined their structures using gas chromatography–mass spectrometry (GC–MS). In this paper, we describe the generation of keto fatty acids, hydroxy fatty acids, and primary fatty acid hydroperoxides from linoleic acid by a pea (*Pisum sativum*) LOX preparation and demonstrate the feasibility of analyzing all of these compounds in a single chromatographic step. We show that our HPLC technique used for this analysis is sufficiently sensitive to permit the investigation of a single pea seed, or part of a seed, which has implications for the genetic studies of LOX activities. We also examine the product profiles generated by enzyme preparations from mutant pea lines in which one or the other of the major LOX polypeptides either is missing or is greatly reduced in amount.

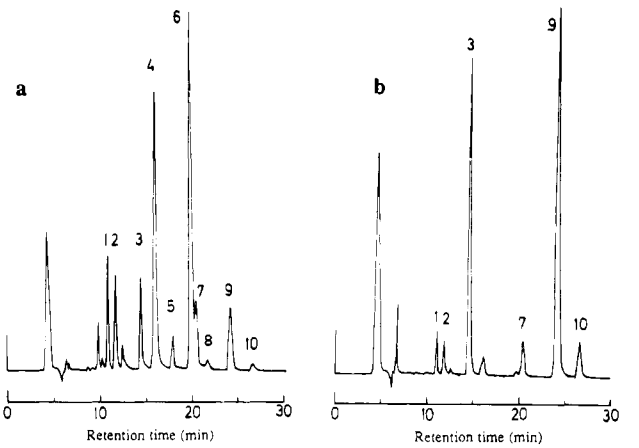

Figure 1. Normal phase HPLC chromatograms of linoleic acid hydroperoxidation products formed by pea (cv. Birte) lipoxygenases (1350 U/mL): (a) Oxygenation products without treatment with sodium borohydride; (b) products reduced with sodium borohydride. Product preparation was as described under Materials and Methods. Peak assignments: 1, 13-KODE; 2, 9-KODE; 3, 13ZE-HODE; 4, 13ZE-HPODE; 5, 13EE-HPODE; 6, 9EZ-HPODE; 7, 13EE-HODE; 8, 9EE-HPODE; 9, 9EZ-HODE; 10, 9EE-HODE. (KODE, keto-octadecadienoic acid; HPODE, hydroperoxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid.)

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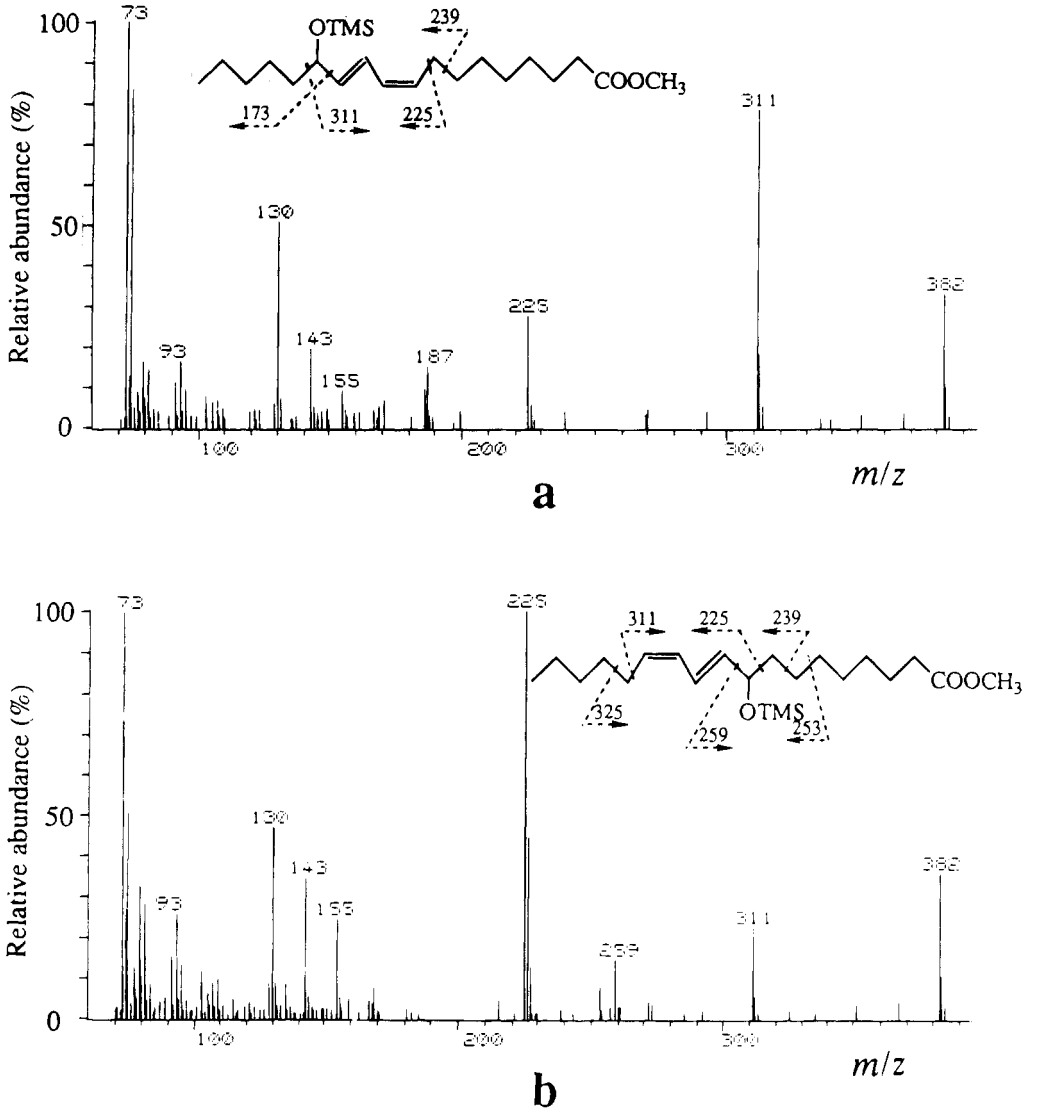

Figure 2. Mass spectra of the trimethylsilyl derivatives of methyl esters of hydroxylinoleic acids: (a) peak 3 (13ZE-HODE) and (b) peak 9 (9EZ-HODE) in Figure 1b.

MATERIALS AND METHODS

Reagents. Linoleic acid, soybean lipoxygenase (type I-B), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane, deuterated chloroform containing 1% tetramethylsilane, and sodium borohydride were from Sigma. 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) was from Aldrich.

Pea Seed Material. Dry mature seeds of three pea (*P. sativum*) lines were used for the preparation of LOX-enriched fractions. The standard line, cv. Birte, contains two major seed LOX polypeptides with predicted amino acid sequences similar to those of LOX-2 and LOX-3 from soybean (Domoney et al., 1990). Line 97P lacks the LOX-2 type polypeptide, whereas line 168B has only about 10–20% of the normal amounts of the LOX-3 type polypeptide; each is derived from John Innes germplasm accessions (JI1006 and JI695, respectively; North, 1989) by crossing and back-crossing to cv. Birte (unpublished results).

Apparatus. Waters 600E HPLC and Waters 486 multiple-wavelength recorder were used at 234 nm, unless otherwise stated, and the chromatograms recorded by a Hewlett-Packard HP3394 integrator. A Zorbax SIL column (250 × 4.6 mm; Fisons) was used for analytical work; it was eluted at 0.7 mL/min with hexane/2-propanol/acetic acid (98:2:0.05 v/v/v). For semipreparative work a Zorbax SIL column (250 × 9.4 mm; DuPont) was eluted with the same solvent but at 3.0 mL/min; only reduced samples (i.e., hydroxylinoleic acids) were injected on this column. The fractions containing keto- and hydroxylinoleic acids were subjected to GC–MS and NMR studies with or without derivatization.

All mass spectra were obtained on a Kratos MS80RF mass spectrometer directly coupled to a Carlo Erba GC. Electron impact (EI) mass spectra were recorded using a standard EI ionization box at 150 °C and an electron energy of 70 eV. GC separation was carried out on a BP1 fused silica (film thickness 0.5 μm) capillary column (30 m × 0.33 mm i.d.) with helium as carrier gas. The GC was kept isocratically at 50 °C for 5 min and then programmed from 50 to 280 °C at 5 °C/min. Data were analyzed on a Data General D'Top DG10 computer system.

NMR spectra were recorded on a Bruker AM-400 NMR spectrometer with a probe of 5 mm at room temperature. Typical spectrometer conditions were spectral width 4807 Hz and memory size 8K data points. All spectra were referred to tetramethylsilane as internal standard (δ = 0.0).

Preparation of LOX-Enriched Fractions from Pea Seeds. Mature dry seeds were ground to a fine powder and extracted at 6 °C by stirring with 10 volumes of 50 mM sodium phosphate buffer, pH 6.8, for 3 h. After filtration through muslin and centrifugation for 10 min at 2 °C and 15000*g*, the material precipitating at 25–60% saturation with ammonium sulfate was collected by centrifugation and dissolved in and dialyzed against 50 mM sodium phosphate buffer, pH 6.8, at 6 °C. Portions of the dialysate (1–1.5 mL, containing 60 mg of protein) were applied to a column (60 × 1.6 cm) of Sephacryl HR S-100 and eluted at 20 mL/h at 6 °C. Fractions of 1.2 mL

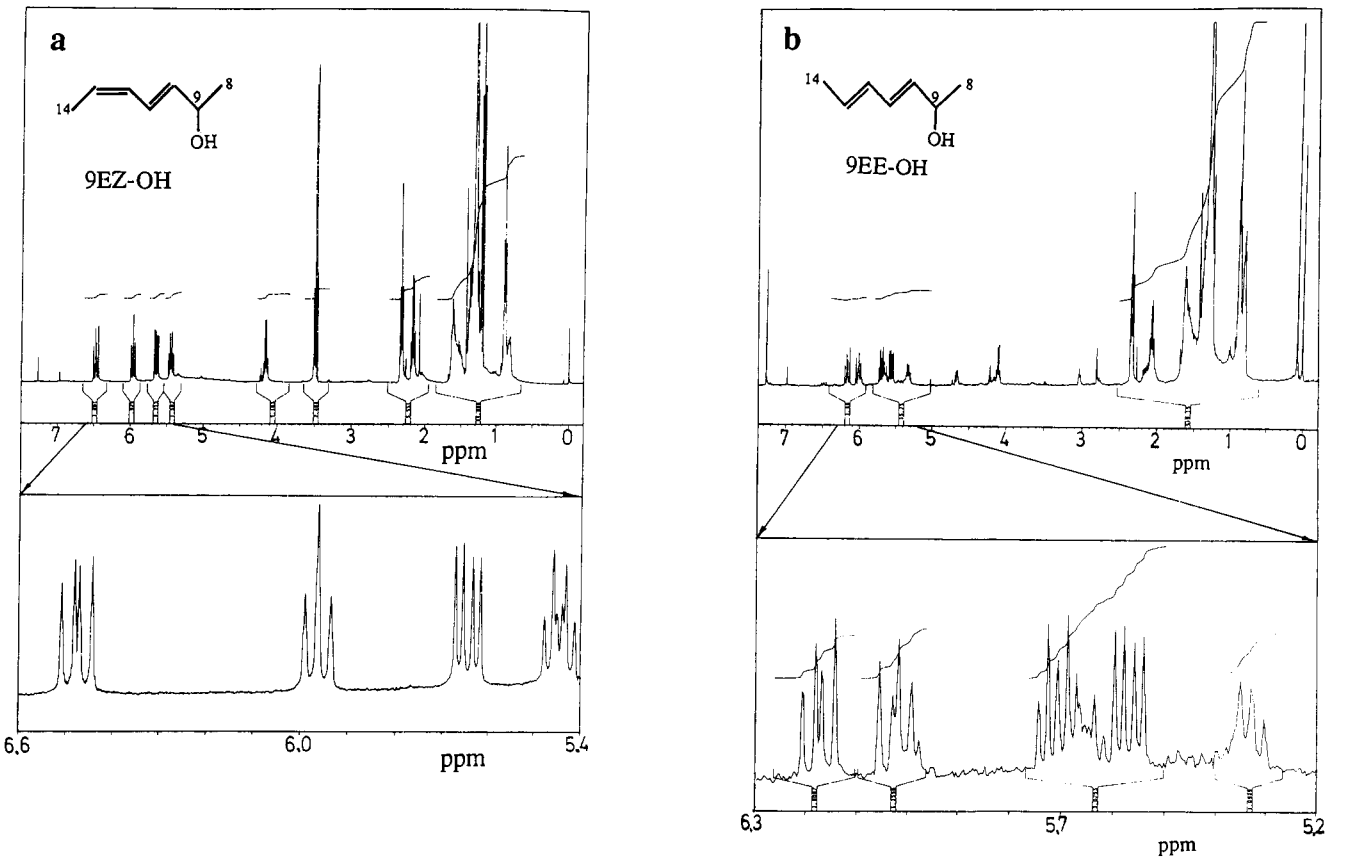

Figure 3. 400 MHz proton NMR spectra of hydroxylinoleic acids in deuterated chloroform with tetramethylsilane as the internal standard (δ = 0.0): (a) peak 9 (9EZ-HODE) and (b) peak 10 (9EE-HODE) in Figure 1b.

were collected and those containing LOX polypeptides identified by SDS gel electrophoresis/Western blotting as described (Domoney et al., 1990). Fractions were pooled for each pea line to give a LOX-active protein preparation containing a similar profile of polypeptides (data not shown).

Preparation of Lipoxygenase Products. The products of the reaction between pea seed lipoxygenases and linoleic acid were formed by incubation of various amount of the enzyme preparation with 1.29 mM linoleic acid in 20 mL of 0.2 M sodium phosphate buffer, pH 6.5, for 15 min under oxygen bubbling at room temperature. The reaction mixture was acidified to pH 2 with dilute hydrochloric acid and extracted twice with 25 mL of diethyl ether. The extract was dried with sodium sulfate, evaporated under vacuum, and redissolved in 3 mL of hexane for HPLC. Hydroxylinoleic acids were prepared by reduction with 100 mg of sodium borohydride for 3 min at room temperature prior to acidification of the reaction mixture.

To identify chromatographic peaks, a mixture consisting predominantly of 13ZE-HPODE was prepared by incubation of 20 mL of soybean LOX-1 [300 units (U)/mL] with 1.29 mM linoleic acid in 0.2 M sodium borate buffer, pH 9.0, for 15 min; other conditions and treatments were as above.

To correlate the hydroperoxides with their reduction products, each HPLC peak was collected, dried under vacuum, and redissolved in 0.3 mL of borate buffer, and 20 mg of sodium borohydride was added. After 3 min at room temperature, the reaction was extracted with 2 mL of diethyl ether, evaporated to dryness, and dissolved in 0.3 mL of hexane for HPLC; retention times were compared to those in the original chromatogram.

Preparation of Trimethylsilylated Linoleic Acids for GC–MS. Fractions that contained hydroxylinoleic acids, from the semipreparative HPLC, were evaporated under vacuum and dissolved in 3.0 mL of diethyl ether/methanol (9:1 v/v). An Aldrich MNNG–diazomethane kit was used to generate diazomethane from the reaction of MNNG with sodium hydroxide at 0 °C (Fales et al., 1973; Black, 1983). The diazomethane reacted immediately with the hydroxylinoleic acids to form methyl esters. When the reaction was complete (~45 min), solvent was evaporated under nitrogen, 0.3 mL of BSTFA containing 1% trimethylchlorosilane was added, and the mixture was incubated for 60 min at 60 °C. The mixture was evaporated under nitrogen, dissolved in 0.2 mL of hexane, and used for GC–MS.

Sample Preparation for NMR. HPLC fractions containing hydroxylinoleic acids were dried under vacuum and dissolved in 0.5 mL of deuterated chloroform containing 1% tetramethylsilane.

RESULTS AND DISCUSSION

Separation of the Pea Seed LOX Reaction Products. When the reaction mixture from the incubation, at pH 6.5, of linoleic acid and the pea seed LOX preparation (total activity 1350 U/mL) was separated on the analytical Zorbax SIL normal phase column, a complex chromatogram was obtained (Figure 1). The unit of the enzyme is defined as that which will cause an increase in absorbance at 234 nm of 0.001/min at pH 9.0 and 25 °C, for a reaction volume of 3.0 mL with a light path length of 1.0 cm, when linoleic acid is used as substrate. Incubation in the absence of enzyme produced insignificant amounts of all products (not shown). Ten of the peaks were given numbers and are identified as follows. Comparison of the patterns before (Figure 1a) and after (Figure 1b) reduction with sodium borohydride shows that peaks 4–6 and 8 decrease on reduction, suggesting that they may be hydroperoxides. The fractions containing these peaks were collected, reduced, and reinjected, showing that they are hydroperoxides and that the correlation between hydroperoxide and hydroxide peaks is 4/3, 5/7, 6/9, and 8/10 (Figure 1). Peaks 1 and 2 also decreased on reduction,

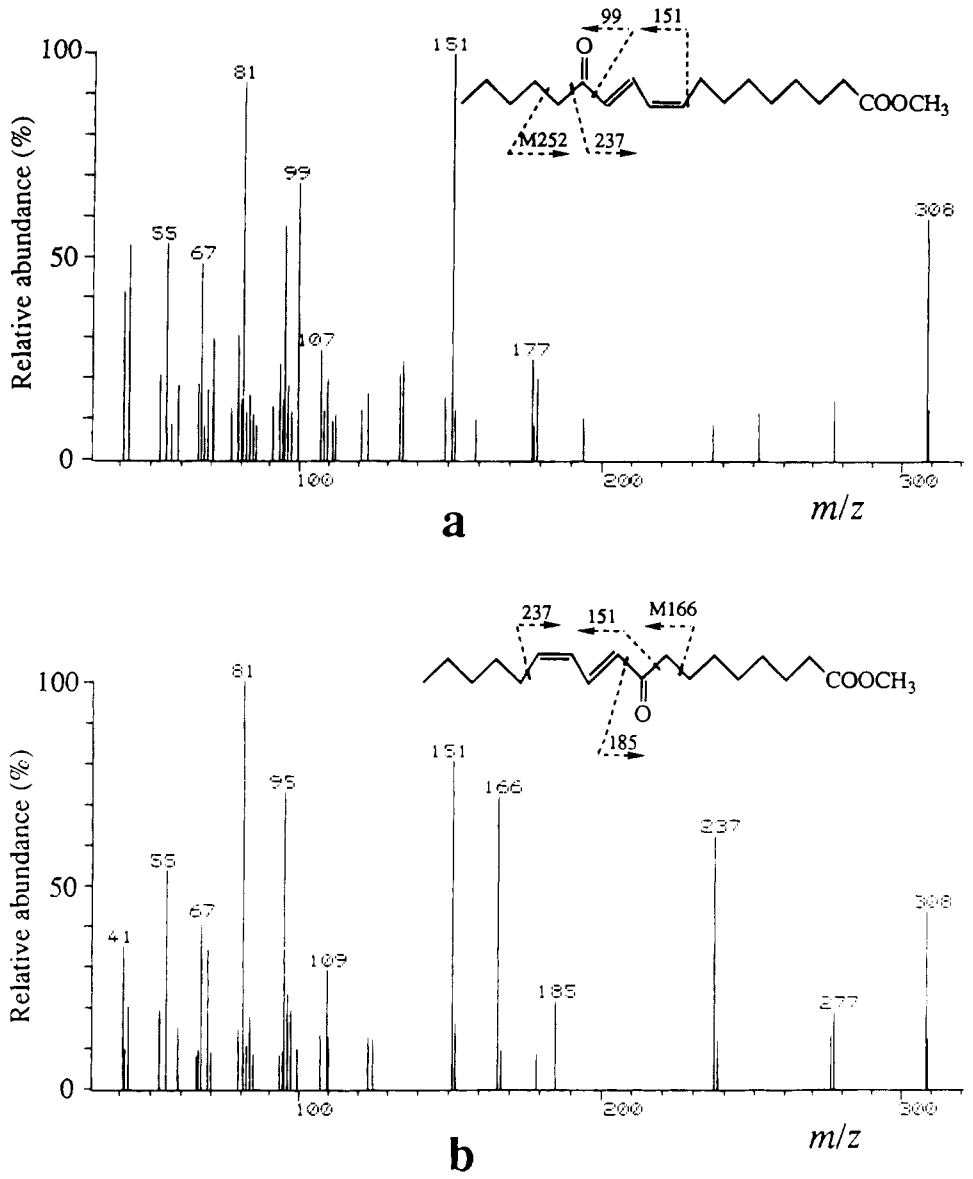

Figure 4. Mass spectra of the methyl esters of the ketolinoleic acids: (a) peak 1 (13-KODE) and (b) peak 2 (9-KODE) in Figure 1b.

but their strong absorbance at 270 nm suggested that they are neither hydroperoxy- nor hydroxylinoleic acids; their identity will be discussed later.

To identify all peaks, the reaction mixture was treated with sodium borohydride and the hydroxides were collected for different derivatizations for GC–MS and NMR experiments.

Identification of 13ZE-HPODE and 13ZE-HODE. Soybean LOX-1 catalyzes the exclusive formation of 13ZE-HPODE when incubated with linoleic acid at pH 9.0 (Roza and Francke, 1973). It is confirmed that peak 4 is 13ZE-HPODE and peak 3 13ZE-HODE by comparing their retention times with those of the exclusive product obtained from the incubation of linoleic acid with soybean LOX-1 and the reduced product (data not shown).

The EI mass spectrum of the trimethylsilyl derivative of peak 3 is shown in Figure 2a. Characteristic peaks can be seen at *m/z* 382 (molecular ion), 367 ($M^+ - 15$; loss of methyl group in the trimethylsilyl group), 351 ($M^+ - 31$; loss of $-OCH_3$), 292 [$M^+ - 90$; loss of $-OHSi(CH_3)_3$, the trimethylsilanol group], 75 [the $-OSiH(CH_3)_2$ group], and 73 [the $-Si(CH_3)_3$ group, the base peak]. The strong peak at *m/z* 311 defines the position of derivatization. The peaks at *m/z* 225 and 173 give the position of the conjugated diene. The peak at *m/z* 173, resulting from the break between C_{12} and C_{13}, would have been stronger than that at *m/z* 311 if the bond between C_{11} and C_{12} had not been a double bond.

Identification of 9EZ- and 9EE-HODE and HPODE. The mass spectra of the trimethylsilyl derivatives of peaks 9 and 10 are similar; Figure 2b shows the mass spectrum for peak 9. The same characteristic peaks of the trimethylsilyl derivatives (see also Figure 2a) are apparent at *m/z* 382, 367, 351, 292, 75, and 73. The strongest (base) peak of peaks 9 and 10, however, is at *m/z* 225, which defines the position of the hydroxy group; the peak at *m/z* 311 defines the position of the conjugated diene. All of this confirms that HPLC peaks 9 and 10 are 9-HODEs; because of the similarity of their mass spectra, the geometric structure of the conjugated diene cannot be identified. To identify the *cis–trans* structure of the bond between C_{12} and C_{13}, NMR spectroscopy was used to determine the spin–spin coupling constants for the protons attached to *cis* or *trans* positions of the double bond.

Table 1. Coupling Constants (Hz) of the Protons Attached to the Double Bonds

	$J_{9,10}$	$J_{10,11}$	$J_{11,12}$	$J_{12,13}$	$J_{13,14}$
peak 9	6.8	15.1	11.0	11.0	7.7
peak 10	7.2	15.2	10.4	15.1	7.2

Figure 3a shows the ^{1}H NMR spectrum of peak 9; the chemical shifts of the protons attached to double bonds are in the range 5.0–7.0 ppm. Spin–spin splitting patterns, decoupling experiments (data not shown), and chemical shifts were used to assign the NMR peaks to each proton attached to the double-bond carbons. Since the *trans* proton has a larger spin–spin coupling constant (J_{trans}) than its *cis* isomer (J_{cis}) (Abraham et al., 1988), the coupling constants from the ^{1}H NMR spectra of peaks 9 (Figure 3a) and 10 (Figure 3b) permit assignation of *cis* and *trans* isomers. The coupling constants are shown in Table 1.

Table 1 shows that the C_{10}–C_{11} bond in both isomers is in the *trans* form, whereas the C_{12}–C_{13} bond in the isomer from peak 9 is in the *cis* form, the coupling constant being smaller than that from peak 10, in which both double bonds are *trans* forms. Thus, peak 9 is 9EZ-HODE and peak 10 9EE-HODE and, therefore, peak 6 is 9EZ-HPODE and peak 8 9EE-HPODE.

Identification of 13EE-HPODE and 13EE-HODE. Only one hydroxylinoleic acid (peak 7) and hydroperoxylinoleic acid (peak 5) remained to be identified. The EI mass spectra of the trimethylsilyl derivatives of peak 7 confirmed it as 13-HODE (data not shown). Therefore, this must be 13EE-HODE and thus peak 5 13EE-HPODE.

Identification of 13- and 9-KODE. The greater absorbance of peaks 1 and 2 at 270 nm (at which wavelength the conjugated diene does not absorb) suggested that they may contain carbonyl compounds. GC–MS of these peaks, given in Figure 4, showed spectra very similar to those reported by Kuhn et al. (1991). Both gave the molecular ion at *m/z* 308 (methyl ester of the ketodienoic acids). In the spectrum of peak 1 (Figure 4a), fragments at *m/z* 99 and 237 define the position of the carbonyl group. The base peak at *m/z* 151 defines the position of the conjugated diene, and the peak at *m/z* 252 is due to the McLafferty rearrangement. In the spectrum of peak 2 (Figure 4b), fragments at *m/z* 151 and 185 define the position of the carbonyl group, the peak at *m/z* 237 defines the position of the conjugated diene, and the peak at *m/z* 166 is due to the McLafferty rearrangement.

Products from LOX Preparations from Standard Compared to Mutant Peas. The LOX-enriched fraction from cv. Birte gave approximately equal amounts of 13ZE-HPODE and 9EZ-HPODE (peaks 4 and 6 in Figure 1a). That from line 97P, which lacks the LOX-2 type of polypeptide, gave 13ZE-HPODE and 9EZ-HPODE in the ratio 1:2 (Figure 5a), whereas that from line 168B, which has greatly reduced amounts of the LOX-3 type polypeptide, gave the same products in the ratio 3:2 (Figure 5b). This indicates that pea seed LOX-2 catalyzes hydroperoxidation predominantly at the 13 position and LOX-3 at the 9 position. Since *n*-hexanal is derived from cleavage at the C_{13} position of 13ZE-HPODE through the action of hydroperoxide lyase, this suggests that off-flavors caused by *n*-hexanal are likely to be associated more with LOX-2 action, as in soybean (Davies et al., 1987).

Analysis of LOX Products from Single Pea Seeds. Figure 6 shows the product profile from the reaction of linoleic acid in the presence of crude extract from a single seed of cv. Birte. The large increase in 13- and 9-HPODEs indicated the presence of LOX activity; the activity at pH 6.5 was higher than that at pH 9.0, consistent with the findings using the LOX-enriched fractions (data not shown). It clearly will be possible to analyze the product profiles from the LOX activity contained in a portion of a seed, which will permit the remainder of the seed to be grown on for subsequent generations. Such a capability is valuable in genetic analyses of LOX activities in segregating populations from breeding programs designed to remove specific LOX activities from pea seeds.

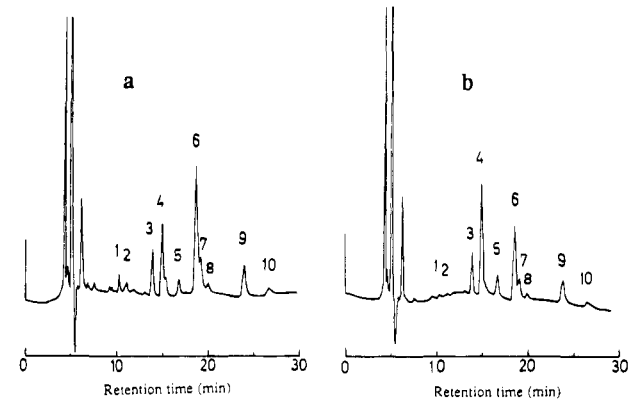

Figure 5. Normal phase HPLC chromatograms of the hydroperoxidation products of linoleic acid formed with lipoxygenases (100 μg of protein) from mutant peas 97P (a) and 168B (b). The peak assignments are as in Figure 1.

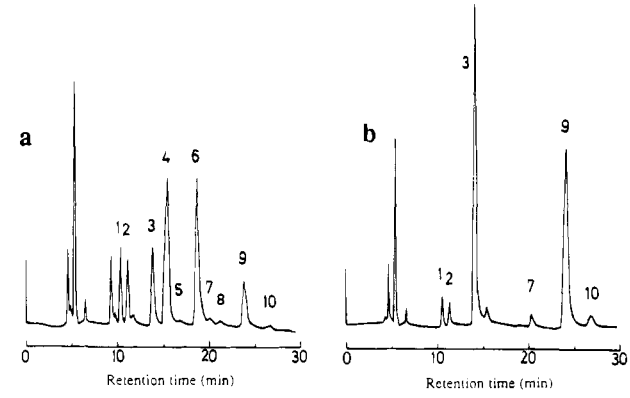

Figure 6. Normal phase HPLC chromatograms of the hydroperoxidation products of linoleic acid formed with a single pea seed of cv. Birte. The peak assignments and (a) and (b) are as in Figure 1.

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