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The positional specificities of the oxygenation of linoleic acid catalyzed by two forms of lipoxygenase isolated from Bengal gram (*Cicer arietinum*)

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Abstract. The products generated from linoleic acid by the two forms of Bengal gram lipoxygenase, BGL_1 and BGL_2, were analysed by high-performance liquid chromatography using μ-porasil column with isooctane containing 0·5% ethanol as the solvent system. The 13-hydroperoxyoctadecadienoic acid and its 9-isomer which are known to be produced by soybean lipoxygenase-1 and the potato enzyme respectively were used as standards. The results show that BGL_1 generated almost exclusively the 13-hydroperoxyoctadecadienoic acid while BGL_2 produced both 13- and the 9-isomer in the ratio 21:79. The secondary keto derivatives formed in the BGL_2 reaction were also separated by this technique.

Keywords. Lipoxygenase; Bengal gram.

Introduction

Lipoxygenase (Linoleate: oxygen oxidoreductase, EC 1·13·1·13) catalyzes the oxygenation of fatty acids containing *cis, cis*-l,4-pentadiene system to form isomeric conjugated dienoic hydroperoxides. The enzymatic production of positional isomers, which, for linoleic acid can be either 9-hydroperoxy-10,12-octadecadienoic acid (9-LOOH) or 13-hydroperoxy-9,11-octadecadienoic acid (13-LOOH) varies with enzyme source, pH, temperature and oxygen level (Galliard, 1975). Much work has been done on the specificity of lipoxygenase from different plant species (Veldink *et al.,* 1977; Galliard and Chan, 1980) as well as of lipoxygenase isozymes from soybean (Axelrod, 1974).

Prior to the development of high performance liquid chromatographic technique (Chan and Prescott, 1975) the separation of the positional isomers was time consum ing apart from its quantitative aspects being questioned (Roza and Arancke, 1973). Since then, high-performance liquid chromatography (HPLC) has been extensively used for the separation of isomeric hydroperoxides (Pattee and Singleton, 1977; Chan and Levett, 1977; Yamamoto *et al.,* 1980; Shoji *et al.,* 1983; Kaplan and Ansari, 1985; Teng and Smith, 1985; Haslbeck and Grosch, 1985).

Here we report on the positional specificities for linoleate oxidation of the two forms of lipoxygenase isolated from Bengal gram.

Materials and methods

Seeds of Bengal gram were obtained from the Seed Corporation of India, Mysore. Linoleic acid was obtained from Nucheck Prep. Inc. Minnes. DEAE-sephadex was

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Abbreviations used: HPLC, High-performance liquid chromatography; TLC, thin-layer chromatography.

purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxylapatite was prepared according to Bernardi (1971). A stock solution of 10 mM sodium linoleate in Tween-20 was prepared as described by Axelrod *et al.* (1981). All other reagents were of analytical grade and the solvents for HPLC were distilled and filtered before use.

Isolation of enzyme

The lipoxygenase in Bengal gram extract was purified by ammonium sulphate fractionation (30-60% fraction) and DEAE-sephadex chromatography. It was resolved into two active forms by hydroxylapatite chromatography. These two forms were designated as BGL_1 and BGL_2 respectively in the order of their elution from the hydroxylapatite column. After rechromatography on hydroxylapatite both the forms appeared to be homogeneous as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The details of the purification procedure and molecular properties of BGL_1 and BGL_2 will be published elsewhere.

The enzyme was assayed either by following the oxygen consumption using a Gilson Oxygraph or by the appearance of conjugated diene hydroperoxide absorbing at 234 nm using a Beckman Model-26 Spectrophotometer. One unit of enzyme was defined as the utilisation of one μmol of substrate or the formation of one μmol of product.

Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Isolation of products

The reaction mixture (100 ml) contained 10 μmol of linoleic acid in 0·2 M phosphate buffer, pH 6·5. The buffers were kept at 4°C and flushed with oxygen before the start of the reaction. The reaction was carried out for 15 min at 4°C with 15 units of either BGL_1 or BGL_2. After acidifying with 2 M citric acid the products were extracted thrice with chloroform: methanol (2:1) mixture. The combined extracts were washed with water until neutral, dried over anhydrous sodium sulphate, the solvent evaporated to dryness *in vacuo* and the products dissolved in diethyl ether. The products were then methylated with diazomethane. After destroying the excess diazomethane with a few drops of 2 M acetic acid, diethyl ether was removed under a N_2 stream and the solvent changed to isooctane. Ultraviolet spectra of the products were taken against isooctane.

Isolation of 9-D- and 13-L-hydroperoxy linoleic acid

The two isomeric hydroperoxides, 9-D- and 13-L- were prepared by aerobic incubation of linoleic acid (10 μmol) with potato extract at pH 6·5 (Galliard and Phillips, 1971) and soybean extract at pH 9·0(Garssen *et al.,* 1971) respectively. The conditions for the enzymatic reactions, isolation of the products and their methylation were similar to those described for Bengal gram lipoxygenase products.

HPLC

Methylated lipoxygenase products were analysed by HPLC using a Waters Associates Liquid Chromatograph (Milford, Massachusetts, USA) equipped with a 6000 A pump, U6K injector and Model 441 absorbance UV-detector. The conditions for HPLC were: column μPorasil (30 cm × 3·9 mm I.D.); mobile phase, 0·5% anhydrous ethanol in isooctane; flow rate, 2 ml/min; detection, 229 nm for hydroperoxides and 280 nm for ketodienes.

Mass spectrometry

The HPLC purified products after removal of solvent under *vacuo* were reduced with $NaBH_4$ in methanol. The reaction mixture was acidified with 2 M acetic acid, extracted with chloroform, and the chloroform evaporated under a stream of N_2. The dried products were kept at -20°C until used for analysis. Mass spectra of the products were recorded with a Hewlett-Packard 5995B GC-MS instrument using the direct insertion probe.

Thin-layer chromatography

The HPLC purified lipoxygenase products were reduced with $NaBH_4$ and then analysed by thin-layer chromatography (TLC) on silica gel G plates using the solvent system petroleum ether (60-80°C):diethyl ether:acetic acid (80:20:1, v/v/v). The spots were visualized by spraying with 5% (w/v) phosphomolybdic acid in 95% (v/v) ethanol followed by heating at 110°C (Garssen *et al.,* 1971).

Results and discussion

The lipoxygenase activity present in Bengal gram was resolved into two forms. The two forms, designated as BGL_1 and BGL_2 were shown to be kinetically distinct (Borthakur and Ramadoss, 1986). BGL_1 catalyzed the oxidation of linoleic acid exclusively to the diene hydroperoxide which absorbs maximally at 234 nm. The BGL_2 reaction produced ketodienes also in addition to hydroperoxide derivative and the product showed absorption maxima at 234 and 278 nm.

In order to determine the regiospecificities of the reactions of BGL_1 and BGL_2, the products were analysed by HPLC. It is known that at pH 9.0 the soybean enzyme generates essentially the 13-hydroperoxyoctadecadienoic acid (Garssen *et al.,* 1971) while the potato enzyme generates predominantly the 9-isomer (Galliard and Phillips, 1971). These two compounds were prepared using crude enzymes from soybean and potato for use as standards. The products from the two enzymes were methylated and chromatographed separately and as 1:1 mixtures. As shown in figure 1A there is an excellent separation of the two isomers with retention times of 11 and 13·6 min respectively for the 13- and 9-isomers.

The solvent system used appeared to be quite comparable to the hexane: ethanol system used earlier for separation of the two isomers (Chan and Prescott, 1975; Pattee and Singleton, 1977). However, it must be mentioned that the ethanol used

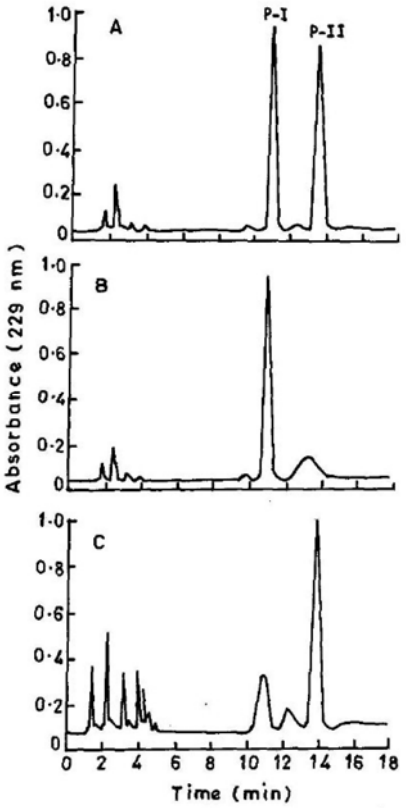


Figure 1. HPLC of methylated linoleate hydroperoxides formed by soybean and potato lipoxygenase (A), BGL_1 (B) and BGL_2 (C) on μPorasil column (300 × 3·9 mm); mobile phase, 0·5% ethanol in isooctane; flow rate, 2 ml/min and UV detection at 229 nm. Component identities: peak-I, 13-LOOH; peak-II, 9-LOOH.

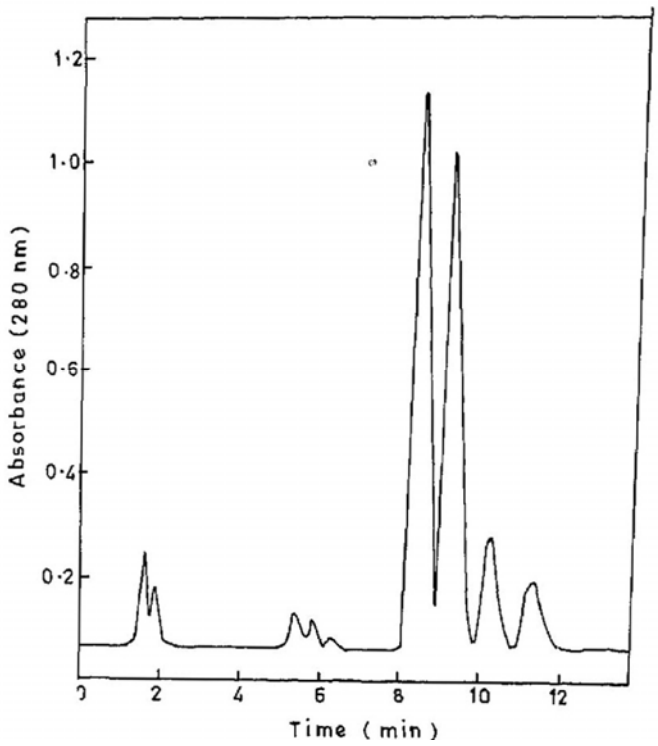
must be anhydrous as the presence of moisture markedly affects reproducibility of the chromatograms.

The proportion of the 13- and 9-isomers were 91:9 and 5:95 respectively for the soybean and potato enzymes and in agreement with earlier reports (Galliard, 1975).

The HPLC analysis of the BGL_1 and BGL_2 products are also shown in figure 1. The BGL_1 reaction product had the same retention time as that of the soybean lipoxygenase product (figure 1B). The minor peak corresponded to the potato enzyme product. Thus, BGL1 generated almost exclusively the 13-hydroperoxy derivative from linoleic acid. In this respect it appears similar to the enzyme from *Dimorphotheca sinuata* which produces only the 13-isomer at pH 6·9 (Gardner *et al.,* 1973). The soybean lipoxygenase-1 generates the 13-isomer exclusively only at pH 9·0; however, at low pH both the isomers are formed (Roza and Francke, 1973).

The HPLC analysis of BGL_2 product (figure 1C) showed the presence of both the 13- and the 9-isomer in the ratio of 21:79. Most of the enzymes having pH optimum below neutrality generate preferentially the 9-isomer (Galliard, 1975; Yamamoto *et al,* 1980).

The keto products generated in the BGL_2 reaction was eluted in this chromatogram as a single peak with a retention time of 4 min. When the ethanol concen-



Figured 2. HPLC of methylated ketodienes of linoleic acid formed by BGL_2. Mobile phase, 0·2% ethanol in isooctane and other conditions as in figure 1.

tration in the mobile phase was reduced to 0·2% the keto derivatives separated into two components (figure 2). In TLC also the ketodiene fraction was found to be a mixture of two closely migrating components.

The UV spectra of the BGL_1 products before and after HPLC separation showed absorption only at 234 nm. The BGL_2 products on the other hand showed two peaks at 234 and 278 nm before HPLC separation. After HPLC separation, the hydroperoxide fraction showed only the 234 nm absorption peak. While the keto derivatives showed absorption only at 278 nm. On $NaBH_4$ reduction of ketodienes the 278 nm peak disappeared but a new peak appeared at 234 nm for the hydroxyl diene chromophore.

TLC comparison of the $NaBH_4$ reduction products of the two ketodienes separated by HPLC with the $NaBH_4$ reduction products derived from soybean, potato and BGL_1 enzyme products indicated that the first major peak (figure 2) is for 13-oxooctadecadienoic acid (figure 3). The second major ketodiene was not obtained completely pure and its reduction product had two spots corresponding to the 13- and the 9-hydroxy derivatives (figure 3). However, it is clear that the two ketodienes isolated from the BGL_2 catalyzed reaction were the 13- and the 9-oxo derivatives. It is likely that they arise from their respective hydroperoxy derivatives, which are the primary products.

The mass spectrum recorded for the HPLC purified soybean enzyme product contained the following informative ions: m/z = 310(M); 208 (M–71; 239–31; loss of $(CH_2)_4CH_3$ and OCH_3); 179 (M–100, 210–31, loss of CHO $(CH_2)_4CH_3$ and OCH_3). The potato enzyme product contained the following ions: m/z=310(M); 279

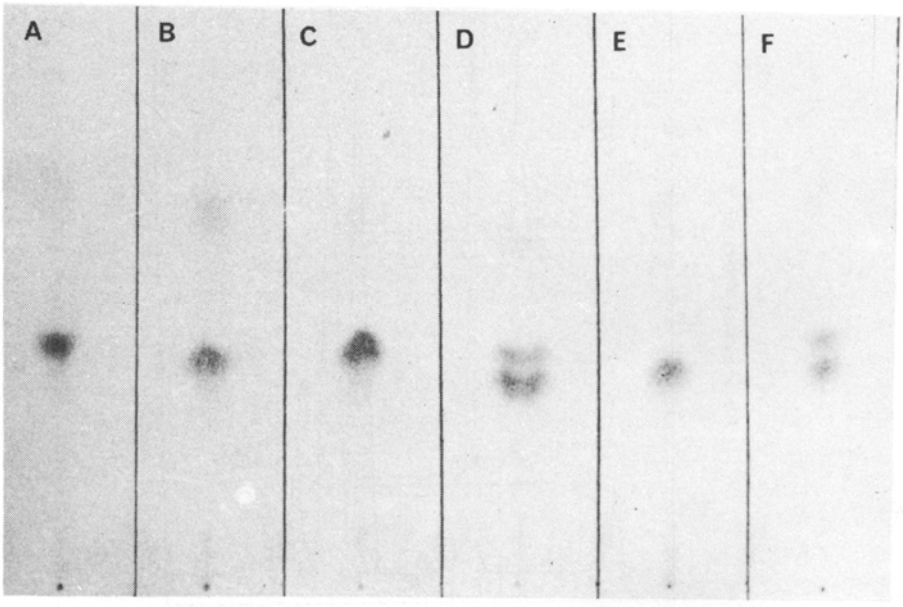


Figure 3. TLC separation of the HPLC purified lipoxygenase products after their $NaBH_4$ reduction. (A), Soybean enzyme product; (B), BGL_1 product; (C), BGL_2 generated ketodiene-I; (D), BGL_2 generated ketodiene-II, (E), potato enzyme product; (F), BGL_2 generated hydroperoxides.

(M–31, loss of OCH_3); 167 (M-143, $(CH_2)_6COOCH_3$); 153 (M-157, $(.CH_2)_7COOCH_3$) and 125 (M-185, $.CHO(CH_2)_7COOCH_3$).

The results confirm the presence of 13- and the 9-isomers of hydroperoxyocta-decadienoic acid in the soybean-L_1 and the potato enzyme catalyzed reactions respectively.

The mass spectra of the BGL_1 product (figure 1B, peak I) and the BGL_2 product (figure 1C, peak II) were identical to those of the soybean-L_1 and the potato enzyme products respectively.

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