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Aerobic Formation of Keto Diene from Linoleic Acid Catalyzed by One of the Two Forms of Lipoxygenase Isolated from Bengal Gram (*Cicer arietinum*)

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Lipoxygenase from Bengal gram (*Cicer arietinum*) has been resolved into two active forms. One of them catalyzes the formation of conjugated diene hydroperoxide from linoleic acid while the other forms keto diene among other products. The aerobic formation of keto diene as one of the major products in a lipoxygenase reaction is unique. This suggests that the two forms of the enzyme present in Bengal gram are distinct.

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12), which catalyzes the hydroperoxidation of fatty acids containing *cis,cis*-1,4-pentadiene system, has been well studied in soybean and shown to exist in multimolecular forms (Axelrod, 1974). They exhibit differences in their pH–activity profile, substrate specificity, and the positional specificity (Axelrod, 1974). These forms have also been resolved by HPLC and shown to be antigenically distinct (Ramadoss and Axelrod, 1982). Multiple forms of this enzyme have been reported in pea (Erriksson and Svensson, 1970; Reynolds and Klein, 1982) and also in a few other species [winged bean (Truong et al., 1982); cowpea (Truong and Mendoza, 1982); wheat (Hsieh and McDonald, 1984); rice (Shoji et al., 1983)].

Some lipoxygenases, besides catalyzing the hydroperoxidation reaction, form other secondary products such as keto diene (Vioque and Holman, 1962). Originally these were shown to be generated only anaerobically (Garssen et al., 1971; Garssen et al., 1972) but were later reported to occur also under aerobic condition (Pistorius, 1974; Hurt and Axelrod, 1977). However, this reaction was not well characterized.

We report here the presence of two forms of lipoxygenase in Bengal gram, one of which catalyzes the aerobic formation of keto diene as a major product. This kinetic feature clearly distinguishes one form from the other.

MATERIALS AND METHODS

Materials and Chemicals. Seeds of Bengal gram were obtained from Seed Corp. of India, Mysore, India. Linoleic acid was obtained from Nucheck Prep. Inc. Minnes. DEAE-Sephadex was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Hydroxylapatite was prepared according to the method of Tiselius as described by Bernardi (1971). A stock solution of 10 mM sodium linoleate in Tween-20 was prepared as described by Axelrod et al. (1981).

Isolation of the Enzyme. Bengal gram flour (50 g) was stirred with 250 mL of 50 mM borate buffer, pH 8.0, for 30 min at 4–6 °C. The extract was centrifuged at 12000*g* for 20 min. The enzyme in the supernatant was purified by ammonium sulfate fractionation and DEAE-Sephadex chromatography. The active fraction from this was resolved into two activities on hydroxylapatite column. The two forms were further purified by rechromatography on hydroxylapatite column followed by molecular sieving on Sephadex G-200. The purified forms appeared to be homogeneous as judged by electrophoresis on SDS–polyacrylamide gel. Details of the purification procedure and molecular properties of the enzyme will be presented elsewhere. The two forms of the enxyme were designated as BGL_1 and BGL_2 according to the order of their elution from the hydroxylapatite column. The final preparations of the enzyme were in 50 mM sodium phosphate buffer, pH 6.8, containing 1 mM EDTA and 40% glycerol and stored at −20 °C.

The enzyme was assayed either by following the oxygen consumption on a Gilson oxygraph or by the appearance of conjugated diene absorbing at 234 nm on a Beckman Model 26 spectrophotometer.

One unit of enzyme is defined as the utilization of 1 μmol of substrate or the formation of 1 μmol of product per minute under the assay conditions.

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

For the determination of K_m for linoleic acid, linear regression analysis of the data was done on Hewlett-Packard HP 33E programmable scientific calculator. Parameters for Hofstee plot were used to get the apparent K_m.

Isolation of Products. Linoleic acid (2 μmol) was incubated separately with 3 units of either BGL_1 or BGL_2 in 20 mL of sodium phosphate buffer, pH 6.5. The reaction was carried out for 4 min at 4 °C. After acidification with 2 M citric acid, the products were extracted twice with 2 vol of hexane–ether mixture (80:20, v/v). The combined extracts were washed with distilled water until neutral.

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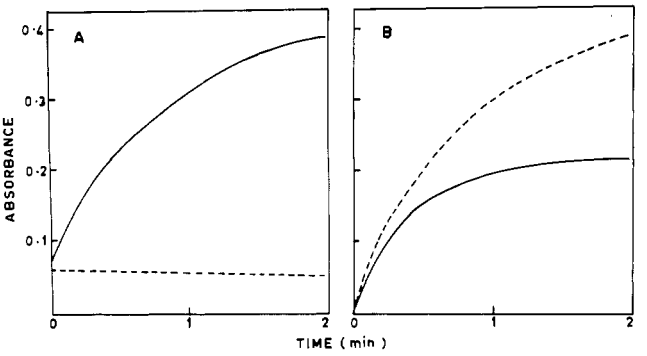

Figure 1. Rate of formation of 234- (—) and 280-nm-(- - -) absorbing products generated from linoleic acid by the action of (A) BGL_1 and (B) BGL_2.

The extract was dried over anhydrous sodium sulfate and then evaporated to dryness in vacuo. The products in methanol were reduced with $NaBH_4$. Ultraviolet spectra of the products were taken before and after reduction.

13-L- and 9-D-hydroperoxyoctadecadienoic acids were prepared by aerobic incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0 (Garssen et al., 1971) and the potato enzyme at pH 6.5 (Galliard and Phillips, 1971), respectively. Hydroperoxides were isolated from the reaction mixture as described for BGL_1 and BGL_2 products. For spectrophotometric measurements, the preformed hydroperoxide in methanol, either 13-L- or 9-D-, was taken in sodium phosphate buffer, pH 6.5. The final methanol concentration was less than 2%. The reaction was started by adding suitable aliquots of BGL_2, and the utilization of the preformed hydroperoxide was observed by recording the change in absorbance at 234 or 280 nm.

Thin-Layer Chromatography. Separation of the reaction products by TLC was performed on 0.25-mm layers of silica gel G coated on 20 × 20 cm plates using a Camag automatic TLC coater. The solvent system used was petroleum ether–diethyl ether–acetic acid (80:20:1, v/v/v). The spots were visualized by spraying with 5% (w/v) solution of phosphomolybdic acid in 96% (v/v) ethanol and heating at 110 °C (Garssen et al., 1971). Peroxides and keto dienes were detected by spraying the plates with ferrous thiocyanate and (2,4-dinitrophenyl)hydrazine, respectively, as described by Vioque and Holman (1962).

RESULTS AND DISCUSSION

The two forms of the enzyme, BGL_1 and BGL_2, isolated from Bengal gram had specific activities of 95 and 130 units/mg of protein, respectively, as determined from the oxygen consumption data at 25 °C. The kinetics of formation of conjugated diene (234-nm absorbance) and that of secondary product (280-nm absorbance) are given in Figure 1. The reaction was carried out at 25 °C in 50 mM sodium phosphate buffer, pH 6.5, containing 100 μM linoleic acid. It is evident that BGL_1 produced exclusively the conjugated diene compound and none of the 280-nm-absorbing product. It may be noted that the rate of the reaction falls off rapidly with time. Similar observations were made with soybean lipoxygenase and attributed to reaction inactivation of the enzyme (Cook and Lands, 1975). This was also evident from the fact that addition of more substrate at the end of 5–7 min of reaction had little influence on the reaction rate, while the addition of another aliquot of enzyme gave yet another burst of activity (results not shown). In the case of BGL_2 there was the formation of 280-nm-absorbing product along with the conjugated diene. The 280-nm-absorbing product was also formed with higher concentrations of substrate (upto 1 mM).

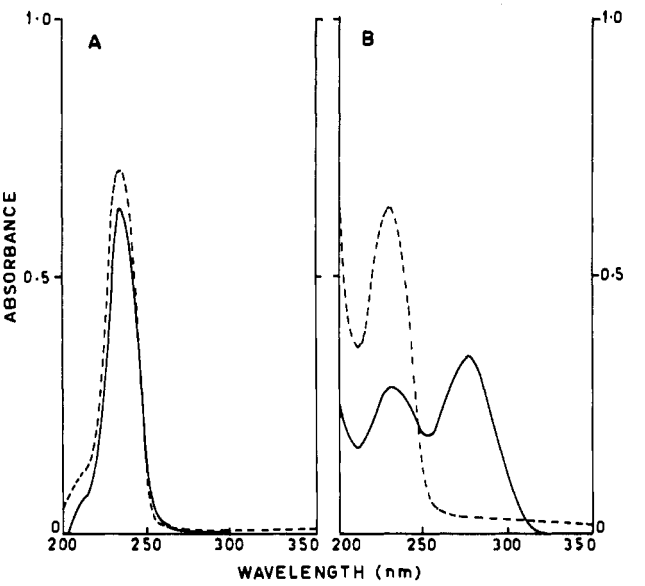

Figure 2. Absorption spectra before (—) and after (- - -) $NaBH_4$ reduction of the products generated from linoleic acid by the action of (A) BGL_1 and (B) BGL_2.

The absorption of lipoxygenase-catalyzed reaction products at 278 nm has been shown to be due to the formation of keto diene (Vioque and Holman, 1962). In order to establish that the 280-nm absorption in this case was due to keto diene formation, we isolated the products from a larger incubation mixture and determined their spectral properties. In Figure 2A is given the spectra of the BGL_1 product before and after reduction with $NaBH_4$. The reduced as well as the unreduced products absorbed maximally at 234 nm. The hydroxy conjugated diene formed upon reduction showed a slight increase in absorbance. The BGL_2 product on the other hand showed a spectrum (Figure 2B) that had two distinct absorbance peaks, one at 234 nm and the other at 278 nm. Upon reduction with $NaBH_4$, the 278-nm peak disappeared completely and the intensity at 234-nm increased. This is attributed to the formation of hydroxy conjugated diene from keto diene (Vioque and Holman, 1962). From the millimolar extinction coefficients of ϵ 25 and 22 reported in the literature (Verhagen et al., 1977), respectively, for conjugated diene hydroperoxide and keto diene, it can be calculated that of the total substrate converted about 50% was the keto diene.

The products were also analysed by TLC on silica gel. In the solvent system used, the unreduced product of soybean L_1 and BGL_1 gave a single major spot beside some unreacted substrate (Figure 3, columns A and B). The product was also identified as a hydroperoxide by its positive reaction (red spot) with ferrous thiocyanate reagent. The BGL_2 product gave several spots (Figure 3, column C), the major spot having a higher R_f value (0.2) than that of BGL_1 or soybean L_1 product (0.17). This spot appears to consist of at least two compounds migrating close to each other. It gave a positive reaction (orange) only with (2,4-dinitrophenyl)hydrazine, suggesting that it is a keto derivative. The reason for the presence of more than one component is not clear at the moment. The spot that had an R_f value (0.13) identical with that of potato lipoxygenase product (Figure 3, column D) gave positive reaction with thiocyanate, indicating it to be a hydroperoxide. Besides the keto and hydroperoxy derivatives there are other products formed in BGL_2 reaction. It is evident that the keto derivative is a major product of this reaction. Further work is needed to characterize these compounds.

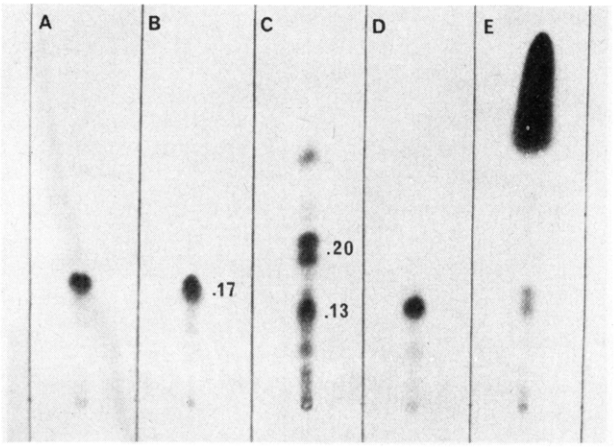

Figure 3. Thin-layer chromatograms of the products generated from linoleic acid by the action of (A) soybean lipoxygenase-1, (B) BGL_1, (C) BGL_2, (D) potato lipoxygenase, and (E) linoleic acid without enzyme. The spots were visualized with phosphomolybdic acid spray. The R_f values are given along with the spots.

Although the primary product of the lipoxygenase reaction is a hydroperoxyoctadecadienoic acid, the formation of keto diene and other secondary products raises the question whether preformed hydroperoxide can serve as substrate for BGL_2 reaction. In order to test this both 13-L- and 9-D-hydroperoxyoctadecadienoic acids prepared from soybean L_1 and potato lipoxygenase, respectively, were used. Both isomers were not converted to 280-nm-absorbing compounds by BGL_2. Similarly, the BGL_1 product also failed to serve as a substrate for BGL_2 reaction. Even in the presence of a small amount of linoleic acid (5–10 μM) there was no conversion of preformed hydroperoxide under aerobic conditions.

The two active forms of lipoxygenase from Bengal gram showed a significant difference in the apparent K_m for linoleic acid (BGL_1 7 μM; BGL_2 80 μM). However, one has to be cautious in interpreting this result because of the complications introduced by the formation of secondary products in the BGL_2 reaction. The pH profiles were similar for both forms, with maximal activity around pH 6.5. The important difference lies in the nature of the products they generate. In its ability to form solely the hydroperoxyoctadecadienoic acid from linoleic acid, BGL_1 appears similar to that of soybean L_2 (Pistorius, 1974) and bush bean (b) form (Hurt and Axelrod, 1977). In this respect BGL_2 falls in the line with soybean L_3 and bush bean (a) form. However, it may be mentioned here that the ability to form keto diene seems to vary a great deal. Thus, in the case of soybean L_3 and bush bean (a) the net conversion of the primary product to keto diene is very low, while BGL_2 converted around 50% of the substrate to keto diene. Preformed hydroperoxy derivatives were not converted to keto diene aerobically by BGL_2. Although such conversion data are not available for soybean and bush bean enzyme, the former was shown to use 13-hydroperoxyoctadecadienoic acid exclusively for cooxidation of carotene (Ramadoss et al., 1978).

The decomposition of hydroperoxyoctadecadienoic acid caused by lipoxygenase, metalloproteins or transition-metal ions has been studied in some detail (Gardner, 1980). According to Gardner (1980) the true enzymatic nature of the decomposition of hydroperoxyoctadecadienoic acid by lipoxygenase is questionable. The BGL_2-catalyzed formation of keto diene (secondary product) showed a hyperbolic response with increasing linoleic acid concentrations similar to the formation of hydroperoxy conjugated diene (primary product) (results not shown). The apparent K_m value of 80 μM for BGL_2 mentioned earlier was obtained from the initial velocity data of the 234-nm absorbance. A similar value was derived when the 280-nm values were used. This seems to suggest that the secondary product in all probability arises from true enzymatic reaction.

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