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PURIFICATION AND CHARACTERIZATION OF TOMATO LIPOXYGENASE

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato; lipoxygenase hydroperoxides; hydrophobic chromatography.

Abstract—Lipoxygenase was partially purified (26-fold) from tomato (*Lycopersicon esculentum*) fruits by ammonium sulphate precipitation and hydrophobic chromatography, and further characterized by disc gel electrophoresis, chromatofocusing and M_r determination. The enzyme had a pH optimum of 6.8, and K_m values for linoleic acid and linolenic acid of 1.42 and 2.60 mM, respectively. The pI was 6.3 and electrophoresis at pH 8.0 revealed a major lipoxygenase band at R_f 0.14. M_r determination gave a value of 97 ± 2K. Incubation of linoleic acid with partially purified enzyme gave a mixture of linoleic hydroperoxides in which the ratio of the 9- to the 13-hydroperoxide isomer was 24:1. The major product was characterized as 9-hydroperoxyoctadeca-*trans*-10-*cis*-12-dienoic acid.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) catalyses the oxygenation of fatty acids containing a 1,4-*cis*,*cis*-pentadiene system to conjugated hydroperoxy acids. Although the existence of this enzyme has been known for more than 40 years, no convincing general role for it in the physiology of the plant has been proposed. Vernooy-Gerritsen *et al.* [1] suggest that lipoxygenase exerts its function in cells at the time that rigorous changes in metabolism take place; namely, at the start of mobilization of reserves in storage tissues and the start of the biosynthesis of chloroplasts. Recent papers [2–4] have contained evidence that lipoxygenase is involved in the final step in the pathway of ethylene biosynthesis, i.e. the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene.

A distinguishing feature of each lipoxygenase is the substitution patterns of the products, the metabolism of which has received much attention in recent years. A number of plant enzymes have been characterized which convert fatty acid hydroperoxides to various oxygenated metabolites [5–7]; among them, jasmonic acid has shown plant growth-regulating properties [8]. The type of components produced by the enzymatic reactions depends on the hydroperoxide isomer, which emphasizes the need for identification of the hydroperoxide isomer(s) produced by the lipoxygenase from the source being studied.

In this paper, we describe the purification and partial characterization of tomato lipoxygenase and the characterization of the hydroperoxide isomers produced from linoleic acid by IR and GC/MS.

RESULTS AND DISCUSSION

The variety and stage of ripening of the tomato showed differences in lipoxygenase activity (preliminary studies). The Rio Grande variety was chosen for further study. The specific activities of the crude enzymate extract from 10 g of fruit at different stages of ripening were as follows: green, 0.06 U/mg; green-orange, 0.13 U/mg; orange-red, 0.20 U/mg; red, 0.16 U/mg. In tomatoes of different varieties similar behaviour had been noted [9–11]. For our work, fruit at the orange-red stage of ripening was chosen.

Purification

The lipoxygenase extract isolated from tomato homogenate by ammonium sulphate (25–70%) precipitation, when dissolved in 12 ml of extraction buffer, had a specific activity of 0.74 U/mg, representing a 4-fold purification and 83% recovery. On Phenyl-Sepharose CL-4B column (1 × 25 cm) chromatography of the extract with an increasing linear gradient of 50% ethylene glycol, lipoxygenase activity appeared in only one peak (Fig. 1). The

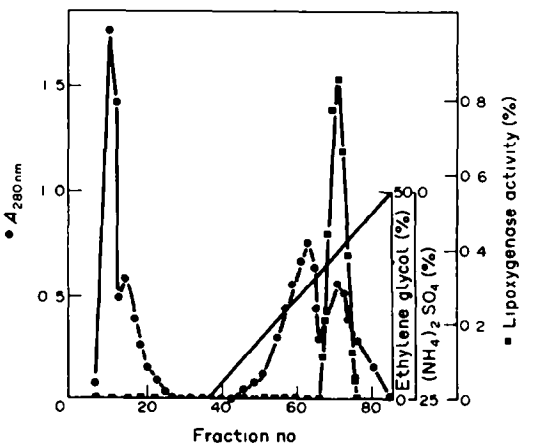

Fig. 1. Hydrophobic chromatography of tomato lipoxygenase.

fractions (67–75) pooled for subsequent enzyme characterization had a specific activity of 4.45 U/mg, i.e. a 26-fold purification with a 70% yield. The purification procedure described is fairly rapid, simple and with only one chromatography step achieves a high yield, substantial purification and a high stability. In fact, the pooled fraction stored at −20° retained 100% activity for 1 month.

Characterization and properties

Disc electrophoresis on polyacrylamide gel at pH 8.0 revealed the presence of one major band at R_f 0.14 and a faint one at R_f 0.36; this weak band may be due to the presence of other lipoxygenase isoenzymes (cf. R_f values reported in refs. [12–15]). Gel chromatofocusing on Polybuffer Exchanger PBE-94 over the pH range 4–8 indicated that the enzyme possessed one pI at pH 6.3. The lipoxygenase exhibited a narrow range of pH activity with a maximum at 6.8; the enzyme had no activity below pH 5.5 or above pH 8.0.

Lineweaver–Burk plots gave apparent K_m values of 1.42 mM for linoleic acid and 2.60 mM for linoleic acid. Substrate inhibition occurred at linoleic and linolenic acid concentrations greater than 0.71 and 0.35 mM, respectively. Gel filtration on Sephadex G-150 indicated a M_r of 97 ± 2K, assuming a uniform spherical shape for the enzyme molecule. Electrophoresis on polyacrylamide gel in the presence of 0.1% SDS and 0.1% mercaptoethanol gave a value of 47K and indicated that the protein was dissociable into two smaller subunits. The partially purified enzyme after 10 min of incubation lost 20% of its activity at 40°, 40% at 50°, and 100% at 60°.

Lipoxygenase isoenzymes are classified as type 1 or type 2 on the basis of their pH of optimum activity, pI and co-oxidative carotene activity [16–18]. Type-2 lipoxygenases generally have a pH optimum of 6–7, a pI of 5.8–6.3 and a high co-oxidative carotene activity. Tomato lipoxygenase is, therefore, type-2 as are most of the lipoxygenases found in plants, and exhibits high co-oxidative carotene activity (unpublished work).

Positional specificity of the hydroperoxides

In characterizing the specificity for the site of O_2 insertion, linoleic acid was used as substrate. Hydroperoxides produced by oxidation with partially purified tomato lipoxygenase were esterified with diazomethane and separated by TLC on silica gel $60F_{254}$ developed with diethyl ether. The hydroperoxides absorbed strongly in UV at 234 nm, and their IR spectra in CS_2 solution had absorption bands at 945 and 985 cm^{-1}, corresponding to *cis–trans* conjugated dienes. HPLC gave the ratio of 13 hydroperoxy-isomer (R_t 17.5 min) to 9-hydroperoxy-isomer (R_t 18.8 min) as 1:24. The peaks had identical retention times (R_t) to the 13- and 9-hydroperoxy-isomers prepared according to the method of Hamberg [19] and Galliard and Phillips [20], respectively.

The final proof of the structures was provided by GC/MS. The components were collected separately from HPLC, reduced with sodium borohydride and hydrogenated (H_2/PtO_2) to yield methyl hydroxystearates. These were then converted into trimethylsilyl ethers. On GC/MS, the TMSi derivative prepared from the presumed 13-isomer gave rise to the expected ions (m/z 173 and 315) due to α,α'-fragmentation of the TMSi group, as did the derivative of the 9-isomer (m/z 229 and 259).

The specificity with regard to the site of O_2 insertion into the substrate is a property of the enzyme although it can be influenced by the experimental conditions. In a more extensive investigation, the effects of O_2 tension, temperature and the presence of Ca^{2+} were studied. Temperature (0, 10, 20 or 30°) had no effect on the ratio of the 13- to the 9-hydroperoxy-isomers. Ca^{2+} (1 mM) increased the lipoxygenase activity by 30% but had no effect on specificity. However, incubations gassed with oxygen yielded 100% of the 9-hydroperoxy isomer.

EXPERIMENTAL

Materials. Tomatoes (cv. Rio Grande) were grown in a greenhouse. They were collected at the orange-red stage of ripening and stored at −20° until required.

Enzyme preparation. Diced tomato (50 g) was homogenized with 1.5 vol. of 50 mM Pi buffer, pH 6.5. The suspension was mixed with the aid of a magnetic stirrer for 30 min at 4°, then centrifuged at 30000 *g* for 15 min. The supernatant was fractionated by $(NH_4)_2SO_4$ precipitation between 25 and 70% saturation. The pellet which sedimented at 15000 *g* for 15 min was resuspended in 12 ml buffer and applied to a Phenyl Sepharose CL-4B column equilibrated with 50 mM Pi buffer, pH 6.5, containing 25% (wt/vol.) $(NH_4)_2SO_4$ and 1 mM EDTA. The proteins were eluted with a linear gradient of 50% ethylene glycol in the equilibrating buffer. Protein determination of the fractions isolated was according to ref. [21] or by absorption at 280 nm.

Enzyme assays. Lipoxygenase activity was determined by measuring the conjugated diene absorption of the hydroperoxide at 234 nm according to ref. [22].

Electrophoresis was performed on 7.5% polyacrylamide gel according to ref. [23]. The run was in 37 mM Tris–glycine buffer, pH 8.9, at 100 V and 50 mA. Proteins were stained with Coomassie brillant blue R-250.

Chromatofocusing was carried out according to ref. [24], using a Polybuffer Exchanger PBE-94 (Pharmacia Fine Chemicals). The pH gradient was formed by Polybuffer 74 on a range 4.0–7.0.

Isolation of products. One ml of lipoxygenase preparation and 1 ml 10 mM linoleic acid soln [22] were added to 30 ml 50 mM Pi buffer (pH 6.8). After 20 min, the mixture was adjusted to pH 3.0 with 2 M HCl, passed through an octadecyl (C_{18}) extraction column from which the products were eluted with MeOH. The conc products were esterified with CH_2N_2, separated by TLC and subjected to HPLC and GC/MS analysis.

HPLC: 22°, Bio-sil HP-10 stainless steel column (4 × 25 mm), 0.75% MeOH in hexane at 1 ml/min, effluent monitored at 234 nm. GC/MS: 25 m OV-1/OV-101 fused silica capillary column, operated isothermally at 200°, electron potential 70 eV.

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