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PURIFICATION AND CHARACTERIZATION OF AVOCADO LIPOXYGENASE

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Key Word Index—*Persea americana*; Lauraceae; lipoxygenase; purification; affinity chromatography

Abstract—Lipoxygenase (E.C.1.13.1.13) from the avocado cultivar 'Fuerte' was purified to near homogeniety by affinity chromatography. The enzyme was extracted in potassium phosphate buffer at pH 7.2 in the presence of 2% Triton X-100. Triton was removed from the homogenate by adsorption on 250–350 mesh activated charcoal. Lipoxygenase was partially purified (seven-fold) by 66% acetone precipitation from a 20% acetone supernatant. The precipitate was dissolved in a potassium phosphate buffer at pH 7.2 and loaded on an affinity chromatography column. This single-step chromatographic purification yielded a single lipoxygenase activity peak. The total activity yield of the purification procedure was *ca* 65% and the degree of enrichment *ca* 35-fold. The M_r determined by gel filtration and by electrophoresis, was 74 000. Optimum enzyme activity was found at 36° and pH 7.1. The energy of activation, amino acid composition, isoelectric point, kinetic parameters and inhibitory effect of epicatechin were studied. The enzyme was found to obey Michaelis–Menten kinetics. The K_m of the avocado lipoxygenase for linoleate was 7.2×10^{-2} mM and the V_{max} was 432 μmol/hr/mg. Epicatechin acted as a competitive inhibitor with a K_i of 9.0×10^{-5} mM.

INTRODUCTION

In the presence of molecular oxygen, lipoxygenase (linoleate: O_2 oxidoreductase E.C. 1.13.1.13.) catalyses the oxidation of C_{18}-unsaturated fatty acids with a *cis,cis*-1,4-pentadiene group to give hydroperoxide [1]. Several conventional and non-conventional methods for the isolation of this enzyme from a variety of plant and animal tissues have been described [2–7]. Affinity chromatography purification was reported by Grossman *et al.* [8] for soybean lipoxygenase, and recently by Cohen *et al.* [9] for that from pea. Attention has been drawn recently to avocado lipoxygenase because of its suggested physiological role in the breakdown of the antifungal 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene involved in the resistance of unripe avocado fruit to fungal infection [10–12]. The present paper describes conditions for the purification of avocado lipoxygenase by affinity chromatography and the catalytic properties of this enzyme.

RESULTS

Cold acetone (4°) was added to the crude enzyme preparation to a concentration of 20% v/v and the precipitate was removed after 1 hr by centrifugation at 20 000 *g* for 10 min. The supernatant was adjusted with additional acetone to 66% v/v and after 2 hr, again centrifuged at 20000 *g* for 10 min. The precipitate was dissolved in 200 ml of 20 mM potassium phosphate buffer, pH 7.2, and the insoluble fraction separated by centrifugation. A 10 ml aliquot of the supernatant was applied to the column of cross-linked Sepharose–hexamethyl–linoleate (10 × 0.7 cm) and eluted with the same buffer at a flow rate of 18 ml/hr. The eluent was collected in 6 ml fractions. Protein from the column was monitored by absorbance at 280 nm.

The elution profile of the crude enzyme preparation from the cross-linked linoleate column showed that most of the inactive protein was eluted with the buffer (Fig. 1). The lipoxygenase activity remained tightly bound to the column even after prolonged washing with the buffer. The bound enzyme was released from the linoleate column with a linear gradient of sodium chloride, 0.0–0.5 M, in the same buffer. Elution commenced when the NaCl concentration approached 0.2 M. Fractions containing lipoxygenase activity were pooled, dialysed against 20 mM potassium phosphate buffer, pH 7.2, for 24 hr, lyophilized and redissolved in 2 ml of distilled water. This solution was used as a purified enzyme source for further study.

The yields, degrees of enrichment and specific activity of the avocado lipoxygenase for a typical purification procedure are summarized in Table 1. In this experiment the specific activity of the purified enzyme was 18.35 μmol O_2/min/mg protein (0.3×10^{-6} Kat/mg) and the enrichment was 35-fold. The purity of the enzyme was evidenced by the single protein band obtained by SDS gel electrophoresis. Cross-linking with ethylene diamine was also explored, but purification by this column was not ade-

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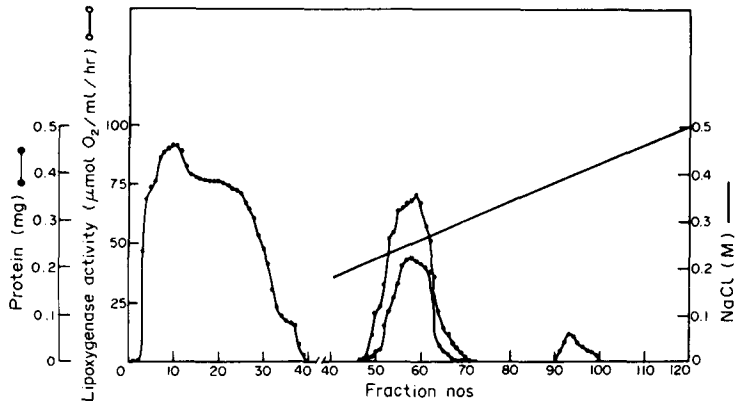

Fig. 1. Purification of the 66% precipitate of avocado lipoxygenase through a chromatography column containing cross-linked Sepharose linoleate. Fractions of 6 ml were collected. After fraction no. 45, an increasing linear gradient was started from 0 to 0.5 M NaCl in 20 mM potassium phosphate buffer (pH 7.2) (——). A_{280} was measured continuously (●—●) and the lipoxygenase activity (○—○) was measured by polarography.

Table 1. Purification of lipoxygenase from avocado peel

STEP	Total protein (mg)	Total activity (μmol O_2/min)	Specific activity μmol O_2/min/mg	Yield (%)	Enrichment
Crude extract	731.2	380.2	0.52	100	1
Acetone 20%	314.6	376.0	1.19	98	2.3
Acetone 66%	82.0	303.5	3.70	79	7.1
Affinity column	13.5	248.4	18.36	65	35.3

The enzyme was assayed by polarography at pH 7.2 with 7.5 mM linoleate in 0.2 M K-Pi buffer containing 0.25% (w/v) Tween 20.

quate, as indicated by the multiple protein bands obtained from SDS runs.

The M_r estimated from SDS gel electrophoresis was 73600 very close to that measured by gel exclusion chromatography (74000) (Fig. 2). Amino acid analysis of purified avocado lipoxygenase showed that there were no carbohydrates in the preparation. The amino acid composition was calculated on the basis of amino acid ratios and a M_r of 74000. The analysis showed 575 amino acid residues per protein molecule and that the enzyme is rich in aspartic and glutamic acids (Table 2). The elution profile obtained upon isoelectric focussing of the purified enzyme exhibited one peak of lipoxygenase activity, with an isoelectric point of 5.9 (Fig. 3).

The energy of activation for purified avocado lipoxygenase, H_a was 12.6 kcal/mol, and the optimum pH was 6.8. The enzyme was inactive below pH 4.5 or above pH 9.0. The apparent K_m estimated from the Lineweaver–Burk linear plot is 7.2×10^{-2} mM, and the apparent V_{max} is 432 μmol O_2/hr/mg (Fig. 4).

The activity of the purified avocado lipoxygenase was

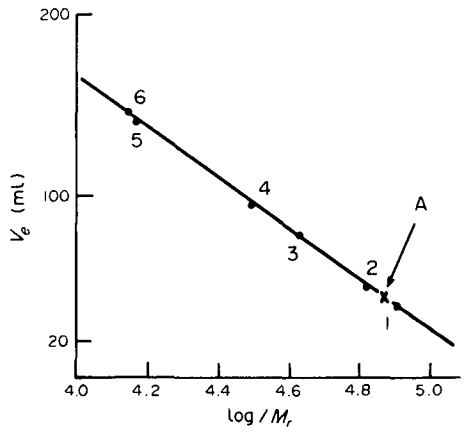

Fig. 2. M_r determination of purified avocado lipoxygenase on Sephadex G-100 filtration. M_r was estimated from a standard plot of elution volume vs log M_r. Standards were 1- alcohol dehydrogenase; 2- bovine serum albumine; 3- ovoalbumine; 4- DNAase; 5- lisozyme; 6- RNAase and A- avocado lipoxygenase.

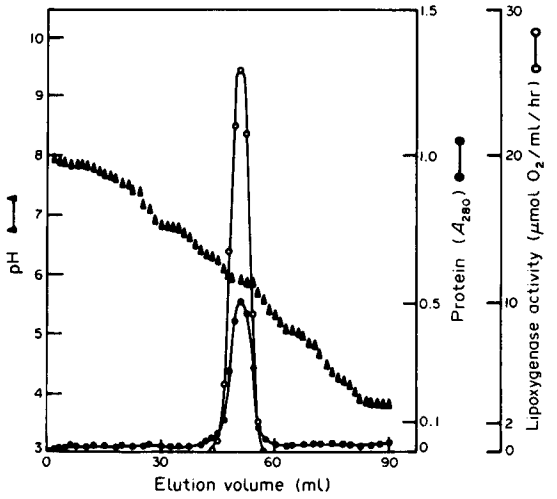

Fig. 3. Isoelectric focussing of purified avocado lipoxygenase. Isoelectric focussing was performed at 4.5° for 3 days. The protein sample was focussed in a pH gradient of 3.5–8.0 at a final ampholyte concentration of 1%. Fractions of 1.5 ml were collected. Protein was determined at 280 nm, activity by monitoring the uptake of O_2 by polarography. The pH (▼) of each fraction was measured at 30°.

inhibited by epicatechin prepared by HPLC-purification from avocado peels cv. 'Fuerte', or by a commercial preparation from Sigma. Epicatechin inhibition was competitive, although the lines converged to a point slightly to the left of the ordinate axis (Fig. 4). The k_i for natural epicatechin, estimated from the double reciprocal plot of enzyme activity versus the linoleic acid concentrations at different concentrations of epicatechin, was 9.0 $\times 10^{-5}$ mM. Similar K_i values were obtained for the commercial preparation of epicatechin.

DISCUSSION

Different lipoxygenases have been isolated and characterized in a large number of plants [8, 9, 13–16]. However, to the best of our knowledge, no study of this enzyme in avocado fruits has been reported. Affinity chromatography purified the enzyme in the Triton extract of avocado 35-fold. Partial purification was obtained with linoleic acid as ligand attached to the gel matrix through an arm of ethylene diamine. However, purification was improved after insertion of a longer arm, hexamethyl diamine, between the Sepharose matrix and the linoleic acid ligand. The interposition of a hydrocarbon arm between gel and ligand was suggested to improve protein-binding capacity in cases involving high M_r proteins, or in systems of intermediate or low affinity (K_i 10^{-2}–1 mM) [17]. Avocado lipoxygenase is specifically retained under these conditions, but can be eluted without further purification, by increasing the NaCl concentration to 0.5 M.

Purified avocado lipoxygenase showed an optimal pH of *ca* 6.8, similar to that reported for some other lipoxygenases, like sunflower [14], wheat germ [1], alfalfa [18], apples [19] and soybean [20]. The isoelectric point, 5.9, is also close to that found for other lipoxygenases: soybean, 5.6–6.2; horse bean, 5.78 and 5.9; and pea, 5.8–6.15 [1].

A comparison of the amino acid composition of avocado lipoxygenase (Table 2) with those from soybean [21], horse bean [22] and pea [23] showed many similarities and few significant differences. The mole percentage of the basic amino acids was similar to that in other plant lipoxygenases [18, 21, 22] and the mole percentage of the acidic amino acid residues was higher than in lipoxygenase from soybean and peas, but similar to that in horse bean. This could explain the isoelectric point of 5.9 for avocado and horse bean, which is more basic than that found for pea (5.8) and soybean (5.65).

The most obvious difference, between avocado and other plant lipoxygenases concerns the sulphur (cystine) and hydroxylic (tyrosine, serine and threonine) amino acid residues. Cysteine and cystine were found in very small amounts in soybean, horse bean and pea lipoxygenases, in contrast to the 15 residues in avocado lipoxygenase. On the other hand, the former lipoxygenases contained more hydroxylic amino acid residues. Only five

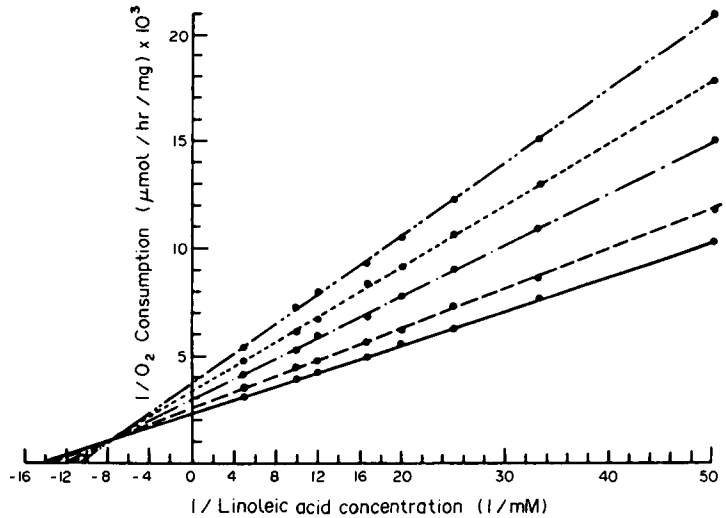

Fig. 4. Reciprocal plot of purified avocado lipoxygenase activity concentration measured by O_2 uptake as a function of linoleic acid only (——), or when supplemented with 4 μg/ml (– – – – –), 10 μg/ml (–·–·–), 20 μg/ml (– – – – –), or 40 μg/ml (–···–···–) epicatechin.

Table 2. Amino acid composition of avocado lipoxygenase

Amino acid	Mol %	Mol/mol*
Asp	11.2	62
Thr	3.3	20
Ser	4.5	32
Glu	10.2	51
Pro	5.6	36
Gly	7.6	75
Ala	6.4	53
Cys	2.4	15
Val	4.9	31
Met	2.8	14
Ile	4.8	27
Leu	8.1	46
Tyr	1.2	5
Phe	3.1	14
His	3.8	18
Lys	7.5	38
Arg	6.6	28
Trp	2.9	10
Ammonia	3.0	130
Total		575
M_r	Gel exclusion	74000
	Gel electrophoresis	73600
Isoelectric point		5.9

*To the nearest integer.

residues of tyrosine per molecule of protein were found in avocado lipoxygenase, and relatively low amounts of threonine and serine.

The M_r of 74000 obtained by gel filtration and by SDS gel electrophoresis is similar to the values found for other lipoxygenases, e.g. 85000 and 66000 for horse bean lipoxygenase [22] and 67000 and 72000 for pea lipoxygenase [23]. The M_r of avocado lipoxygenase is smaller than that of soybean lipoxygenase, *ca* 100000, [21, 24] that apparently consists of two subunits of equal M_r [21]. We have not observed any dissociation of the avocado lipoxygenase during electrophoresis in SDS-gels after treatment of the protein with mercaptoethanol.

The apparent K_m values of avocado lipoxygenase for linoleic acid at pH 6.5 was 7.2×10^{-2} mM. This value is lower than that obtained for type-2 pea lipoxygenase at pH 6.8, which ranged from 2.22 to 3.62 mM, and for pea-seed lipoxygenase at pH 9.0, which ranged from 1.8 to 2.0 10^{-1} mM but it is close to the value of 8.5 10^{-2} reported for soybean lipoxygenase-1 at pH 9.0 [25].

The concentration of the lipoxygenase-inhibitor epicatechin, in unripe avocado fruits, was 514 μg/g fr wt of peel (*ca* 385 μg/ml) and decreased during ripening to 8 μg/g fr wt [12]. Our results show that epicatechin inhibited avocado lipoxygenase competitively, with a K_i of 9.0×10^{-5}. These results suggest that the decrease in epicatechin concentration during ripening of avocado fruit might result in enhanced lipoxygenase activity of these fruits [11].

EXPERIMENTAL

Materials. Avocado fruits, cv. 'Fuerte', were obtained from a commercial orchard in Rehovot, Israel. Activated charcoal (250–350 mesh), Triton X-100, linoleic acid (99% pure), oleic acid and epicatechin were purchased from Sigma Chemical Company. Other chemicals were of analytical grade. CNBr-activated Sepharose 4-B were obtained from Pharmacia.

Extraction of lipoxygenase from avocado peels. Avocado fruit peel (20 g) was homogenized in 200 ml of 5 mM K-Pi buffer, 2% v/v Triton X-100, pH 7.2, at 0°, in a Sorval Omnimixer operated at max. speed for 2 min. The extract was filtered through a single layer of Miracloth and centrifuged at 20000 *g* for 15 min. To the cold supernatant, 15.5 g of activated charcoal (250–350 mesh) was added; the mixt. was stirred for 20 min to remove detergent and centrifuged again at 20000 *g* for 10 min [26]. The supernatant served as a crude enzyme prepn.

Purification of lipoxygenase by affinity chromatography. Affinity chromatography on linoleyl aminohexamethyl sepharose was performed according to the method of ref. [8] which was modified by using CNBr-activated Sepharose 4-B instead of agarose, and hexamethylene diamine instead of ethylene diamine as an arm. The linoleyl-hexamethylene-sepharose cross-link was obtained by coupling of amino-hexamethylene-sepharose with linoleic acid in the presence of 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide [17, 27].

Lipoxygenase assay. Lipoxygenase activity was measured polarographically by monitoring the uptake of O_2 with a Y.S.I. oxygen monitor model 53 in a 5 ml reaction vessel fitted with a Clark-type O_2 electrode. The assays was performed at 30° in 0.2 M KPi buffer (pH 7.2) and initiated by addition of 8 mM linoleic acid emulsified in 0.25% (v/v) Tween-20 according to the method of ref. [27]. The control emulsion was prepared in the same way, using oleic acid. Emulsions were stored under N_2 at 4° in the dark for no longer than 10 days. Enzyme activities were calculated from initial rates of O_2 uptake. The initial dissolved O_2 concn. was determined relative to O_2-satd H_2O. Enzyme activity was expressed as μmol O_2 consumption/min or hr/ml of reaction medium.

Protein was determined in the presence of Triton X-100 by the method of ref. [28] and in its absence by the method of ref. [29].

M_r and isoelectric focussing determination. M_r was determined by gel filtration on a Sephadex G-100 column (75 cm × 2 cm) using 10 mM K-Pi buffer, pH 7.2 [30], and by SDS polyacrylamide gel electrophoresis [31]. Electrophoresis was performed at room temp. for 3 hr, at 200 V and 5 mA. Proteins were stained with Coomassie Brilliant Blue. The isoelectric focussing expts were conducted at 4.5° in a 110 ml column (LKB Type 8101). A density gradient of saccharose was superimposed on the pH gradient using a gradient-mixing device. Expts were performed with Ampholines carrier ampholytes (LKB), with a pH range 3.5–8.0, at a final concn of 1%. After dialysis against 1% glycine soln, 3 ml samples were added to the dense soln after a quarter of the gradient had formed. A constant power of 1.2 W was applied for 3 days; initial and final voltage were 400 V. The bottom electrode served as the cathode. When focusing was completed, the column was emptied at a flow rate of *ca* 0.5 ml/min. Each 1.5 ml fraction was measured for pH, *A* at 280 nm and lipoxygenase activity.

Amino acid analysis. Samples of the purified enzyme were thoroughly dialysed, first against 100 mM KCl and then against deionized H_2O. After lyophilization, a portion was hydrolysed three times in glass-dist. 6 N HCl at 110° in a sealed evacuated tube. Analyses was performed in duplicate after 70 hr of hydrolysis with an automatic amino acid analyser using the method of ref. [32]. Tryptophan was determined spectrophotometrically [33].

K_m, V_{max} and inhibition determination. For the determination of apparent K_m and V_{max} of avocado lipoxygenase at pH 6.5, and 30°, linoleic acid concns were varied from 0.02 to 0.25 mM.