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Regulation of *Colletotrichum Gloeosphorioides* Latency in Avocado

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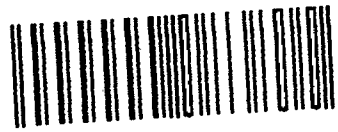

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REGULATION OF COLLETOTRICHUM GLOEOSPORIOIDES LATENCY IN AVOCADO.

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C. ABSTRACT

The resistance of unripe avocado fruits to attack by postharvest pathogens was associated with the preformed antifungal compound called diene. Previous results led to the belief that diene decrease was a result of increased lipoxygenase activity. Activity of this enzyme was regulated in the fruit peel by the decrease in concentra tion of the flavan 3-ol inhibitor, epicatechin and not due to changes in the enzyme protein content. The differential decrease of epicate chin concentration after harvest was used as a criteria for classify ing resistance of avocado cultivars to postharvest disease. Exogenic compounds that inhibited lipid peroxidation activity of lipoxygenase were tested as possible inhibitors of diene peroxidation and were found to daly the development of anthracnose and stem end rot.

D. INTRODUCTION AND ORIGINAL OBJECTIVES.

Colletotrichum gloeosporioides infections of avocado are the cause of the vast majoritys of losses due to fruit decay in Israel and most foreign markets. Colletotrichum spores germinate on the surface of the unripe fruit and produce appressoria, but further development of the fungus is delayed until the fruit ripens after harvest. The resistance of unripe avocado fruits peel to fungal development was found by Prusky et al. to result from the presence of the antifungal compound 1-acetoxy- 2-hydrox y-4-oxo-12,15-heneicosas-diene (1). The concentration of this com pound in avocado peels decreased during ripening, concomitantly with resumed growth of previoulsy quiescent Colletotrichum infections.

Since the antifungal diene shares the cis,cis-1,4-pentadiene sys tem of linoleic acid, the possibility that the diene may be a sub strate for plant lipoxygenase and that lipoxygenase may be respon sible for decomposition of the antifungal compound during avocado fruit ripening was investigated. It was observed that lipoxygenase activity extracted from fruit peel increased by 80% during ripening, coincident with increased respiration and evolution of ethylene, and followed by the apperance of fungal disease symptoms (2). It was observed as well that a partially purified fraction of avocado lipox ygenase oxidized the antifungal diene from avocado in vitro suggest ing that lipoxygenase might be involved in the breakdown of the anti fungal diene. The question that was asked was how is lipoxygenase activity regulated during fruit ripening. Experiments with some lipoxygenase inhibitors suggested the possibility that the decrease of the diene compound in avocado fruits during ripening may be linked to increased lipoxygenase activity (2). Tocopherol acetate infiltra tion of whole fruits inhibited lipoxygenase actvity in extracts from the fruits and delayed the development of disease symptoms in the fruit in vivo. Furthermore, α-tocopherol acetate and butylated hydroxy anisole inhibited lipoxygenase-mediated oxdiation of the diene compound by 42 and 89% respectvely , in vitro. Tocopherol acetate, however, did not inhibit growth of the fungus in culture, indicating that its effect on decay development is via plant metabol ism, probably as an antioxidant.

Based on that initial data, the objectives of the original research were:

1. The isolate natural inhibitor(s) of avocado lipoxygenase and determine the possible relationship with the latency of Colletotri chum gloeosporioides.
2. To determine if the changes of lipoxygenase-inhibitor concen tration or either the increase in specific activity of the enzyme are regulating the breakdown of the antifungal diene.
3. To determine the relation of inhibitor concentration and cul tivar susceptibility to C. gloeosporioides after harvest.
4. To evaluate antioxidants as possible agents for prevention of decay development during fruit ripening and under prolonged storage

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Prusky, D., N. T. Keen, and I.L. Eaks, 1983. Further evidence for the involvement of a preformed antifungal compound in the latency of Colletotrichum gloeosporioides in unripe avocado fruits. Physiological Plant Pathology 22: 189-198.

E. REPORT

1. Isolation of natural inhibitors of avocado lipoxygenase and their relationship with the quiescence of C. gloeosporioides.

Physiological Plant Pathology (1985) **27**, 269–279

Inhibitors of avocado lipoxygenase: their possible relationship with the latency of *Colletotrichum gloeosporioides*

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Linoleate oxidation by avocado lipoxygenase was inhibited *in vitro* in the presence of the specific inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA). Infiltration with ETYA of avocado discs inoculated with *Colletotrichum gloeosporioides* delayed symptom development at concentrations where the fungus itself was not affected. Subsequently, a natural inhibitor of avocado lipoxygenase was isolated from peels of unripe avocado fruits and identified as epicatechin. It inhibited avocado lipoxygenase with a K_i of 0·64 μM. The concentration of epicatechin in unripe fruits was 514 μg g^{-1} fresh weight of peel; this decreased during ripening to 8 μg g^{-1} fresh weight, before symptoms of *C. gloeosporioides* infections were expressed. A comparison of two cultivars with differing susceptibility to *C. gloeosporioides* showed that the concentration of epicatechin decreased faster in the cultivar in which symptoms appeared first. An atmosphere containing 50 μg l^{-1} ethylene enhanced the decrease of the lipoyxygenase inhibitor in avocado fruits and shortened the period before symptoms of disease were expressed. In over-mature, firm and naturally infected fruits hanging in the orchard the concentration of epicatechin was 260 μg g^{-1} in the area of the peel without symptoms and only 27 μg g^{-1} in that showing symptoms of infection.

The results are discussed in relation to the hypothesis that the latency of the infection of avocado fruit by *C. gloeosporioides* may be accounted for by the degradation of the preformed antifungal compound, *cis,cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene, which is catalysed by avocado lipoxygenase, and that the *in vivo* lipoxygenase activity may increase during ripening owing to the decline in the levels of its endogenous inhibitor epicatechin.

INTRODUCTION

Colletotrichum gloeosporioides infects avocado fruits during the growing season but the infections remain superficial and latent until fruit ripening [2]. The lack of involvement of nutrients in the latency of *C. gloeosporioides* was demonstrated in discs of fruit

Contribution from the ARO, No. 1179-E, 1984 series.

Abbreviations used in text: BHT, butylated hydroxy toluene; DMSO, dimethylsulphoxide; ETYA, 5,8,11,14-eicosatetranoic acid; PPE, partial purified extract.

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peel bearing latent infections where disease development was not enhanced by the infiltration of nutrients [*11*]. A preformed antifungal compound was isolated from the peel of unripe avocado fruits and identified as *cis,cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene [*13*]. In unripe peel, this compound is present at fungitoxic concentrations and decreases to a non-fungitoxic level during fruit ripening, concomitant with renewed growth of the fungus and symptom expression. It was suggested that this compound is responsible for the latency of *C. gloeosporioides* infections of avocado fruits. Preformed antifungal compounds have been considered as a possible explanation for latent fungal infections in other fruits [*10, 14, 18*].

Lipoxygenase was shown to catalyse the oxidation of the antifungal diene in an *in vitro* system, and the specific activity of lipoxygenase in avocado peels increased by 80% during fruit ripening [*12*]. The same work gave indications of the possible function of lipoxygenase in decreasing the concentration of the antifungal diene during fruit ripening by introducing α-tocopherol which is known to inhibit lipoxygenase *in vitro* and which delayed development of disease symptoms in the fruit.

The present investigation was designed to explore further the system involved in the degradation of the preformed antifungal diene in avocado and its relationship to latency of *C. gloeosporioides*.

MATERIALS AND METHODS

Ripening indicators and symptoms expression in whole fruits

Fruits of avocado, *Persea americana* Miller var. *drymifolia* (Schlect. and Chamb.) Blake, from trees of the cvs Fuerte and Reed, were obtained from a commercial orchard in Rehovot, Israel. Ethylene and CO_2 evolution and the degree of symptom expression by *C. gloeosporioides* Penz. were determined as described previously [*6, 13*]. Firmness of avocado fruits during ripening was determined by recording the force (kg) required to penetrate the flesh with a conic probe (5 mm diameter and 7 mm length). The average value from six determinations on three fruits from both sides of the longitudinal axis is reported.

Effect of lipoxygenase inhibitors on Colletotrichum development in avocado discs

Discs, 22 mm in diameter and 7 mm thick, were randomly sampled from the surface of freshly harvested cv. Fuerte fruits. The discs were vacuum infiltrated with either a suspension of 0·1 μM butylated hydroxy toluene (BHT, Sigma) or 0·05 μM 5, 8, 11, 14 eicosatetraynoic acid (ETYA) (Ro-3-1482, La Roche, Nutley, NJ, U.S.A.) in an aqueous solution of 2% ethanol, 1% dimethylsulphoxide (DMSO, BDH Chemical Co.) and 0·1% Triton X-100. Control discs were infiltrated with the ethanol, DMSO and Triton X-100 only. Infiltration was accomplished by immersing the discs in the suspension and reducing the pressure to 45 mm Hg for 90 s. The amount of the solution infiltrated was $3{\cdot}2 \pm 0{\cdot}11$ g $(100\text{ g})^{-1}$ fresh weight avocado discs. After infiltration, the discs were disinfected with 1% NaOCl for 2 min and washed twice with sterilized water. Five discs were placed peel side up in a Petri dish, 15 discs per treatment. A 2% agar solution in 0·2 M sodium phosphate buffer, pH 7·2, containing 0·025% syntomicetin and 10 μg μl^{-1} thiabendazole was added to half the height of the discs. Each disc was then inoculated with a 5 μl drop of *C. gloeosporioides* spore

suspension (approx. 10^6 spores ml^{-1}) placed on its centre. Dishes were kept at 20 °C until symptoms appeared. Symptom development was checked using a binocular microscope. Experiments were performed at least twice with each cultivar.

Endogenic lipoxygenase inhibitor

Extraction. Peel (1–2 mm thick) was taken from avocado fruits immediately after harvest or from fruit stored at 20 °C and 20 g were homogenized in 100 ml of 5 mM sodium phosphate buffer, pH 7·2, in a Sorval Omnimixer at full speed, for 2 min, at room temperature. The extract was centrifuged at 20 000 **g** for 10 min and the supernatant acidified to pH 4·5 with concentrated HCl. The acidified supernatant was fractionated twice with ethyl acetate (1 : 1, v/v). The organic phases were pooled and washed with water acidified with HCl (pH 4·5, 1 : 1, v/v); the organic phase was dried with anhydrous $MgSO_4$ and then concentrated to dryness *in vacuo* at 40 °C. The lipoxygenase inhibitory compound was isolated from the organic phase by flash chromatography on a column of silica gel 60 (15–40 μm, Merck) on a 4·3 cm diameter Buchner funnel with a fritted glass disc. The silica gel was poured into the funnel to a depth of 4·2 cm, as described previously [*13*], and the column was equilibrated with dichloromethane. The dried organic phase was dissolved in 100% ethyl acetate, brought to 30% ethyl acetate in dichloromethane and then applied to the column. Successive washes with 200-ml portions of 100% dichloromethane, 10% and 30% ethyl acetate in dichloromethane removed a considerable amount of inactive material. All the active compound(s) was then eluted with 100% ethyl acetate, dried *in vacuo*, dissolved in water (1 ml $10\,g^{-1}$ initial fresh weight) and used as the "partial purified extract" (PPE). The various fractions obtained during separation were checked for the presence of the active compound(s) by measurements of lipoxygenase inhibition and TLC.

Chromatography. Two methods were employed: (a) aluminium-backed cellulose sheets (0·1 mm thick, Merck) without fluorescent indicator, were developed with the upper phase of butanol : acetic acid : water (40 : 10 : 50, v/v); (b) silica gel GF 254 plates (0·375 mm thick, Merck), developed with methanol : chloroform : acetic acid (40 : 60 : 1, v/v).

Purification. The PPE was further purified by HPLC on a 1 × 25 cm, 10 μm LiChrosorb RP-18 column (Merck) and eluted with 20% water in methanol and 0·5% H_3PO_4. The HPLC column was monitored at 275 nm and active peaks were collected.

Chemical identification. The highly purified compound obtained from the reversed phase HPLC column was examined by UV and IR spectroscopy and by proton and carbon magnetic resonance. Ultraviolet data were obtained on a Perkin Elmer model 202 and IR data on a Perkin Elmer model 137 spectrophotometer, and NMR data was acquired on a Varian EM390 (proton) or a JEOL FX200 (proton and carbon) instrument. Acetylation was performed by dissolving 10 mg of purified inhibitor in 400 μl pyridine and allowing it to react with 50 μl of freshly distilled acetic anhydride at room temperature. The acetate was purified by HPLC on a Partisil M9 column with 50% ethyl acetate in hexanes after removal of excess reagent by ether/water partitioning.

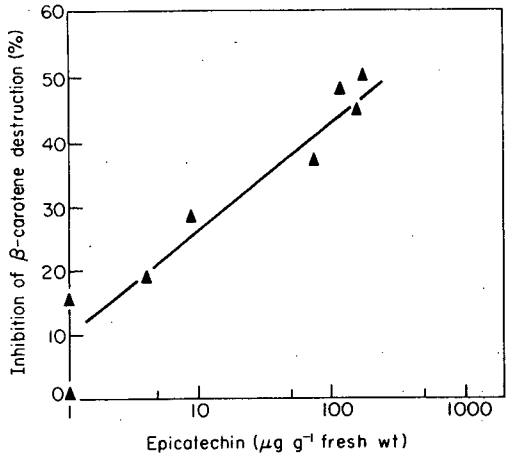

FIG. 1. Relationship between inhibition of lipoxygenase catalysed β-carotene destruction (enzymatic assay for epicatechin concentration) and HPLC purified epicatechin concentration.

Quantitative analysis. In different experiments two methods for quantification of the amount of the inhibitor present in the avocado peels were used.

(a) *HPLC assay*: 10 μl aliquots of the PPE were chromatographed on the RP-18 HPLC column, as already described. Known amounts of the purified inhibitor were used as standards in separate runs. Calculations of the concentrations of the inhibitor were based on the integration of the 275 nm HPLC peak areas of the compound and standards.

(b) *Enzymatic assay*: the inhibitory activity in the intermediate fractions and in the PPE was assayed by determining the inhibition of lipoxygenase-catalysed oxidation of β-carotene in the presence of linoleate [7]. Lipoxygenase activity was determined by colourimetric measurements according to Grossman *et al.* [7]. The reaction mixture contained 1·8 ml of carotene-linoleate substrate in phosphate buffer, pH 7·2, 0·2 M [1], 0·1–0·2 ml (250–500 units) of soybean lipoxygenase (Sigma, grade V) and 20 μl of the PPE, representing 100 mg of peel tissue. Inhibition of lipoxygenase was determined by comparing the rates of carotene destruction during the initial 3 min of the reaction.

In most experiments the amount of inhibitor was assayed by HPLC as well as the enzymatic method; in one experiment the enzymatic method was employed alone. Fig. 1 shows the relation between inhibitor (epicatechin) content of peels as obtained by HPLC and lipoxygenase inhibition by aliquots of the PPEs.

Assay for avocado lipoxygenase and its inhibition by ETYA. Peel of avocado fruits (1–2 mm thick) was extracted at various stages after fruit harvest as previously described [11]. Lipoxygenase activity was determined by monitoring the consumption of O_2 in the presence of linoleate with a YSI Biological Oxygen Monitor, according to the method of Grossman *et al.* [7]. For the determination of inhibitory activity, 0·15 ml of a 6·6 mM solution of ETYA in ethanol was added to the reaction mixture.

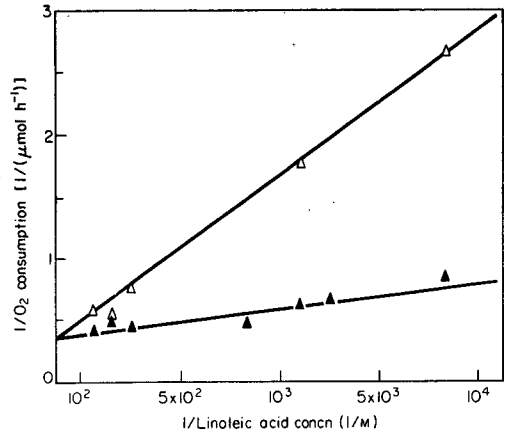

FIG. 2. Effect of eicosatetraynoic acid on the crude dialysed avocado cv. Fuerte lipoxygenase; reciprocal plot of activity (O_2 consumption) *v.* substrate (linoleic acid) concentration. ▲, Linoleic acid only; △, with $6{\cdot}6 \times 10^{-3}$ M eicosatetraynoic acid.

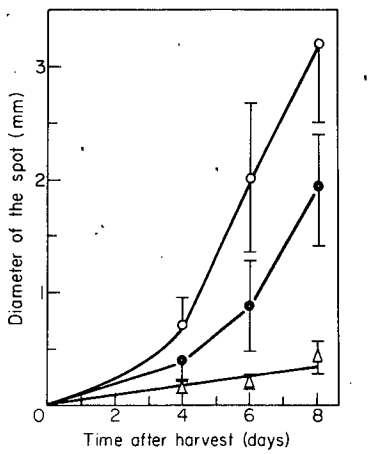

FIG. 3. Diameter of *Colletotrichum gloeosporioides* spots on inoculated avocado cv. Fuerte discs treated with eicosatetraynoic acid (△), and butylated hydroxy toluene (●), or untreated (○). Fruit discs were infiltrated with a suspension of 5×10^{-5} M eicosatetraynoic acid or 10^{-4} M butylated hydroxy toluene prepared in 4% ethanol, 1% dimethyl sulphoxide and 1% Triton X-100; control discs were infiltrated with solvent only. Bars indicate standard deviation.

RESULTS

In vivo *and* in vitro *inhibition of avocado lipoxygenase by ETYA and BHT*

Crude extracts from avocado peel exhibited lipoxygenase activity as observed previously [*11*] with a K_m linoleate of 0·11 mM and a V_{max} of 2·16 mmol O_2 h^{-1} (Fig. 2). The addition of ETYA competitively inhibited linoleate oxidation and resulted in an apparent K_m of 0·37 mM (Fig. 2).

Avocado discs were vacuum infiltrated with 50 μM ETYA or 100 μM BHT. As a result of infiltration with each of the compounds, symptom expression of *C. gloeosporioides* was delayed relative to control discs (Fig. 3). No significant differences were

observed in the firmness of the discs 8 days after inoculation, when significant differences in the development of symptom expression were recorded. Up to 1·0 mM ETYA did not affect germination of *Colletotrichum* on 2% agar.

Purification and identification of an endogenous lipoxygenase inhibitor in avocado peel

The TLC runs of the organic phase (see Materials and Methods) extracted from avocado fruits cv. Fuerte revealed a spot with lipoxygenase inhibitor activity at R_F 0·53 or 0·76 when developed with butanol : acetic acid : water (40 : 10 : 50, v/v) or methanol : chloroform : acetic acid (40 : 60 : 1, v/v), respectively. Following purification by flash chromatography only a single spot appeared; this spot contained the inhibitory activity. The inhibitor(s) could be detected as reddish or blueish spots when sprayed with diazonium reagent or potassium hexacyanoferrate (III)-iron (III)-chloride, respectively. The active fraction obtained by flash chromatography was further purified by HPLC and yielded one major active peak with a retention time of 4·41 min at a flow rate of 3 ml min^{-1}.

The purified compound showed UV absorption at 215 nm ($\varepsilon = 24\,100$) and 280 nm ($\varepsilon = 3350$) in ethanol, and its IR spectrum indicated the presence of hydroxyl and aromatic groups but no carbonyl: (KBr) = 3·0, 6·6, 6·9, 7·35, 7·8, 8·75, 9·15, 9·4 and 9·6 μm. The proton nuclear magnetic resonance spectrum in d_6-acetone exhibited only four aliphatic protons [2·80 dt $J = 5,18$ (2 H); 4·2 br t (1 H); 4·9 s (1 H)], five aromatic protons (5·93 d $J = 2·5$ (1 H); 6·03 d $J = 2·5$ (1 H); 6·85 s (2 H); 7·06 s (1 H)], and a large broad peak containing D_2O-exchangeable protons at 5·2 δ. Only two couplings were observed: a 2·5 Hz *meta* coupling between aromatic protons at 5·93 and 6 03, and a 5 Hz coupling between the CH_2 at 2·8 and the CH at 4·2. Irradiation of the methine at 4·9 caused no change in the rest of the spectrum. Acetylation caused at 1·2 ppm a downfield shift of the methine originally at 4·2, indicating a secondary alcohol at this position. This data could be explained by a cyclic moiety X-CHY-CH(OH)-CH_2Z if X and Z were aromatic and Y an ethereal oxygen. The purified acetate contained four nearly equivalent acetate methyl groups at 2·23δ and one acetate methyl at 1·80 δ, derived from the five exchangeable protons of the natural product, whose formula must be $C_{15}H_{14}O$, comprising two aromatic and one aliphatic ring. At this point, we recognized the structure of this lipoxygenase inhibitor as a member of the catechin family. The ^{13}C NMR spectrum of the natural product in DMSO-d_6 supported this conclusion: 28·4, 65·0. 78·3, 94·4, 95·3, 98·8, 115·0, 115·0, 118·3, 130·9, 144·6, 144·6, 156·0, 156·3, 156·6. This data could be duplicated with a commercial sample of epicatechin examined under the same conditions. Commercial epicatechin was also found to have the same HPLC behaviour as the natural inhibitor.

Epicatechin inhibition of lipoxygenase

The inhibition of lipoxygenase activity by commercial epicatechin (Sigma) or a HPLC-purified extract from avocado cv. Reed were compared. Inhibition of lipoxygenase was determined by the enzymatic assay measuring β-carotene oxidation. Epicatechin was added at five concentrations between 0·02 and 2·0 μM. The K_i was calculated from the abscissa intercept of a Dixon plot [3] of the data obtained from replicate experiments. The K_i values for the natural and commercial epicatechin were 0·64 and 0·71 μM, respectively.

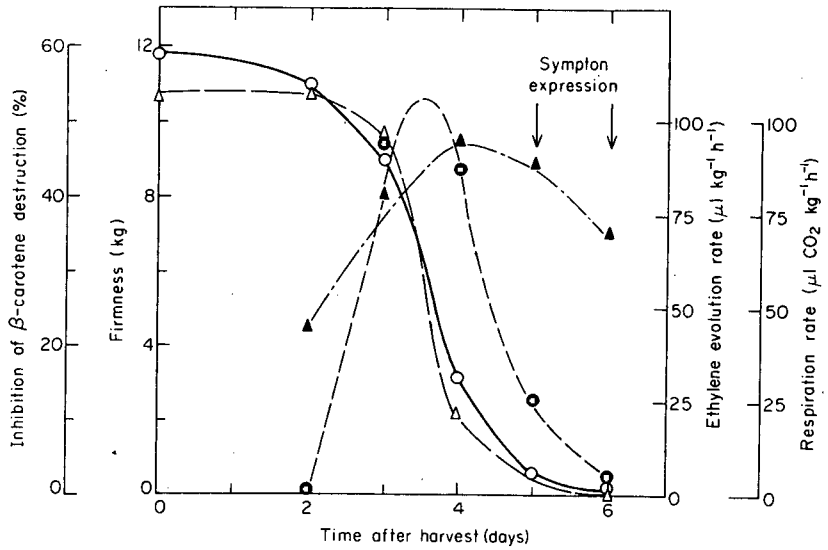

FIG.4. Inhibition of lipoxygenase catalysed β-carotene destruction (△—enzymatic assay for epicatechin), evolved ethylene (●), respiration (▲) and fruit firmness (○) in avocado fruits cv. Fuerte stored at 20 °C.

Relation between ripening process, presence of epicatechin and symptom expression

Partially purified extracts were prepared from the peel of avocado cv. Fuerte at various stages of ripening after storage at 20°C. The concentration of epicatechin in the PPE was estimated by the enzymatic assay. The PPEs of freshly harvested fruits inhibited soybean lipoxygenase activity by 53% (Fig. 4). Four days after harvest, the inhibition decreased to about 10% and when disease symptoms were already present, no inhibition of lipoxygenase was found in the PPEs. Fruit firmness decreased in parallel with the decrease in lipoxygenase inhibition. The most rapid reduction of both occurred after the climacteric peak of ethylene production.

Effect of treatments on ripening, epicatechin concentration and symptom expression

Ethylene. The firmness of freshly-harvested avocado fruits stored at 20 °C decreased from 10·4 kg to 2·3 kg after 7 days and to 0·2 kg after 11 days (Table 1). Storage of such fruits in air containing 50 $\mu l\, l^{-1}$ ethylene accelerated the softening to 1·1 kg 3 days after harvest. Epicatechin concentration in the peel of the fruits was measured by the HPLC assay: in controls it decreased from 137 $\mu g\, g^{-1}$ to 43 $\mu g\, g^{-1}$ fresh weight during the first 3 days of storage and to 8 $\mu g\, g^{-1}$ fresh weight after 7 days. In ethylene-treated fruits, epicatechin decreased to 4 $\mu g\, g^{-1}$ fresh weight after 3 days. Disease symptoms appeared after 11 days in control fruits, while in ethylene-treated ones symptoms of disease were already present 7 days after inoculation.

CO_2-enriched atmosphere. When avocado fruits were sealed in plastic bags they produced an atmosphere of 9% CO_2 and 13% O_2 which prevented fruit softening. Under such storage conditions the progress of avocado softening was compared to the progress of epicatechin disappearance, which was measured enzymatically and to the develop-

TABLE 1

Firmness, epicatechin concentration in the fruit peel and symptom expression of Colletotrichum gloeosporioides *during storage of avocado fruits cv. Fuerte in the presence of ethylene*

Time after harvest (days)	Normal ripening			C_2H_4-treated[a]		
	Epicatechin concn ($\mu g\ g^{-1}$ fresh wt)	Fruit firmness (kg)	Symptom appearance[b] (% infected spots)	Epicatechin concn ($\mu g\ g^{-1}$ fresh wt)	Fruit firmness (kg)	Symptom appearance[b] (% infected spots)
1	137	10·4	0	135	10·4	0
3	43	9·9	0	4	1·1	0
7	8	2·3	0	1	0·3	100
11	1	0·2	100	—	—	—

[a]Harvested fruits were treated with 50 μl^{-1} ethylene at 25 °C for 48 h and then transferred to 20°C.

[b]Darkening of the peel over an area 5 mm in diameter was considered to indicate an expression of infection.

TABLE 2

Effect of storage of avocado fruit cv. Fuerte in CO_2 enriched atmosphere[a] on firmness, inhibition of β*-carotene destruction (enzymatic assay of epicatechin concentration) and* Colletotrichum gloeosporioides *symptom expression*

Treatment	Days after harvest	Inhibition of β-carotene destruction (% of control)	Symptom appearance (% infected spots)	Fruit firmness (kg)
Control	0	86·4	0	11·5
	7	0·0	100	0·2
CO_2 enriched	7	15·5	20	9·1
	9[b]	0·0	100	7·5

[a]Atmosphere was composed of 9% CO_2 and 13% O_2.

[b]After 7 days in CO_2 enriched atmosphere, fruits were transferred for an additional 2 days to air.

ment of symptoms (Table 2). During 7 days of storage, firmness decreased only from 11·5 to 9·1 kg, lipoxygenase inhibition by epicatechin in the PPEs decreased from 86 to 15·5% and only 20% of the inoculated spots showed disease symptoms. In control fruits, in a normal atmosphere, disease symptoms appeared on 100% of the infected sites after 7 days; during the same time the inhibition of lipoxygenase decreased to zero and firmness to 0·2 kg.

When the treated fruits were removed from the plastic bags, all the inoculated spots showed decay symptoms within 48 h, concomitant with a total disappearance of lipoxygenase inhibition activity in the PPEs. However, fruit firmness at this time was still 7·5 kg. This latter result shows that symptom appearance is not directly related to ripening, and is better correlated with the disappearance of lipoxygenase inhibition.

TABLE 3

Firmness, epicatechin concentration in the fruit peel and symptom expression of Colletotrichum gloeosporioides *during normal ripening at 20 °C of two avocado cvs Fuerte and Reed*

	Fuerte			Reed		
Time after harvest (days)	Epicatechin conc ($\mu g\ g^{-1}$ fresh wt)	Fruit firmness (kg)	Symptom appearance (% infected spots)	Epicatechin (concn ($\mu g\ g^{-1}$ fresh wt)	Fruit firmness (kg)	Symptom appearance (% infected spots)
1	137	10·4	0	514	13·3	0
3	43	9·9	0			
5				171	12·0	0
7	8	2·3	0			
9				104	10·3	0
11	1	0·2	100			
12				163	7·7	0
17				71	0·5	0
20				8	0·4	100

Behaviour of different avocado cultivars

The firmness of cv. Fuerte fruits decreased from 10·4 to 0·2 kg 11 days after harvest (Table 3). During the same period, the concentration of epicatechin decreased from 137 to 1 $\mu g\ g^{-1}$ fresh weight and disease symptoms were recorded when epicatechin attained the latter level. In cv. Reed fruits, firmness was still 7·7 kg 12 days after harvest and it decreased to 0·5 kg only 5 days later. Accordingly, the epicatechin concentration in Reed fruits was still 163 $\mu g\ g^{-1}$ fresh weight 12 days after harvest and decreased to 8 $\mu g\ g^{-1}$ fresh weight only after 20 days, when disease symptoms appeared as well.

Epicatechin and decay symptoms in the orchard

Sometimes, apparently as a result of wounding, symptoms of *Colletotrichum* infection appear on Fuerte fruits while these still hang on the trees. This occurs particularly in overmature fruits. The firmness of such fruits, 4–5 months after their regular harvest time was still 11 kg (avocado does not ripen and soften while still on the tree). The epicatechin concentration in the peel of the healthy parts of these fruits was 260 $\mu g\ g^{-1}$ fresh weight, while it was only 27 $\mu g\ g^{-1}$ fresh weight in the decayed area of the same fruits.

DISCUSSION

In previous work the preformed antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,14-diene, was isolated from avocado fruit peels. This compound seems to be responsible for the latency of *C. gloeosporioides* infections [*13*].

The possibility that avocado fruit lipoxygenase is related to the degradation of the antifungal diene during ripening when *Colletotrichum* becomes active was supported by the previously reported results [*12*] showing that (i) the apparent specific activity of fruit lipoxygenase increases by 80% during ripening; (ii) the avocado antifungal diene

serves as a substrate for lipoxygenase of avocado and soybean; and (iii) treatments with α-tocopherol acetate, an antioxidant which inhibits lipoxygenase [*4,12*] delayed the decrease of the antifungal diene as well as the appearance of disease symptoms [*12*].

In the present work, a specific inhibitor of lipoxygenase, ETYA, as well as an antioxidant of lipoxygenase, BHT, was used to demonstrate the involvement of the enzyme in the diene degradation. ETYA is an instantaneous and irreversible inhibitor which acts as a substrate analogue [*4, 16, 17*], while BHT functions as a free radical trap [*5*]. Both compounds inhibited avocado lipoxygenase *in vitro* and delayed the development of disease symptoms in fruit discs (*in vivo*) without affecting their ripening and decrease in firmness.

An endogenous inhibition of lipoxygenase has now been isolated from peels of avocado fruits and identified as epicatechin. This antioxidant was previously isolated from avocado by Masako & Naoko [*8*]. Purified epicatechin from the peel of avocado fruit competitively inhibited lipoxygenase-catalysed oxidation of β-carotene in the presence of linoleate with a K_i of 0·64 μM, similar to that of commercial epicatechin.

Several lines of evidence obtained in this investigation support the hypothesis that a decline in levels of epicatechin may be responsible for the increase of lipoxygenase activity during ripening, the resulting degradation of the antifungal compound and the appearance of symptoms of infection by *C. gloeosporioides*.

(i) Epicatechin levels decreased, from values of 140 to 500 $\mu g\, g^{-1}$ fresh weight of peel in unripe fruits to 8 $\mu g\, g^{-1}$ fresh weight in ripe and symptoms expressing fruits.

(ii) Fuerte fruits exhibited disease symptoms after a shorter storage period than Reed fruits and the concentration of epicatechin also decreased more rapidly during storage of the former cultivar.

(iii) Ethylene treatments enhanced fruit ripening as well as the decrease in epicatechin concentration and appearance of symptom expression.

(iv) A CO_2 enriched atmosphere, which delayed the degradation of epicatechin, also delayed symptom appearance.

(v) Firm, unripe but overmature fruits still hanging on the trees had an overall high concentration of epicatechin, but contained decayed areas with low epicatechin concentration.

In conclusion, previous results [*11, 12, 13*] supported the hypothesis that the preformed antifungal diene in the peel of unripe avocado fruits confers resistance to *C. gloeosporioides* and that the degradation of this diene may result from lipoxygenase activity. The present data further indicated that lipoxygenase and the degradation of the antifungal diene may be regulated in avocado peel via lipoxygenase inhibition by the flavan-3-ol epicatechin, acting as a trap for free radicals [*5, 9, 15*].

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2. Involvement of epicatechin in the regulation of antifungal diene concentration during activation of quiescent Colletotrichum gloesporioides infections of ripening avocado fruits.

This study was designated to test wheter an increase in lipoxyge nase activity during ripening indeed results from degradation of its endogenous inhibitor epicatechin or from an increase of the amount of enzyme in the fruit. The use of a immunological technique for quanti tation of lipoxygenase in crude extracts was developed and for this purpose was needed the purification to homogenity of avocado lipox ygenase. We report here the process of purification of avocado lipox ygenase and the use of an immunological technique to determine the relationships among lipoxygenase activity, lipoxygenase amount and its inhibitor during fruit ripening.

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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 20000 *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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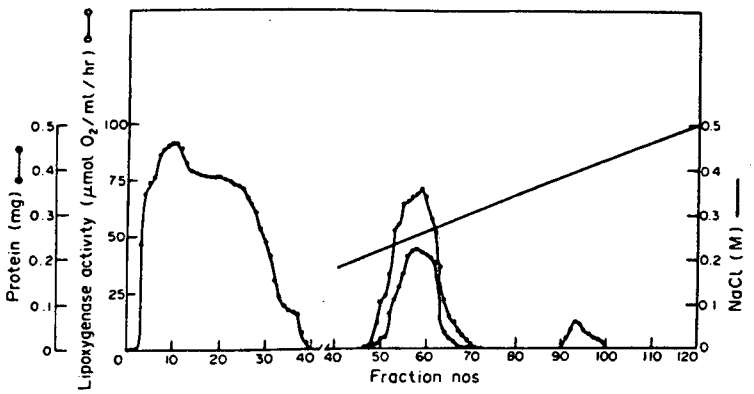

Fig. 1. Purification of the 66% precipitate of avocado lipoxygenase through a chromatography column containing cross-linked Sepharose linoleate. Fractions of 6 ml were collected. After fraction no. 45, an increasing linear gradient was started from 0 to 0.5 M NaCl in 20 mM potassium phosphate buffer (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 73 600 very close to that measured by gel exclusion chromatography (74 000) (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 74 000. 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

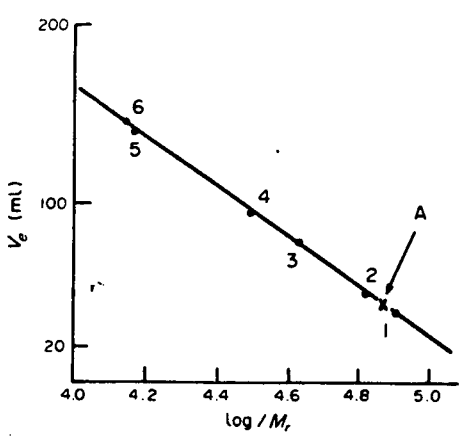

Fig. 2. M_r determination of purified avocado lipoxygenase on Sephadex G-100 filtration. M_r was estimated from a standard plot of elution volume vs log M_r. Standards were 1- alcohol dehydrogenase; 2- bovine serum albumine; 3- ovoalbumine; 4- DNAase; 5- lisozyme; 6- RNAase and A- avocado lipoxygenase.

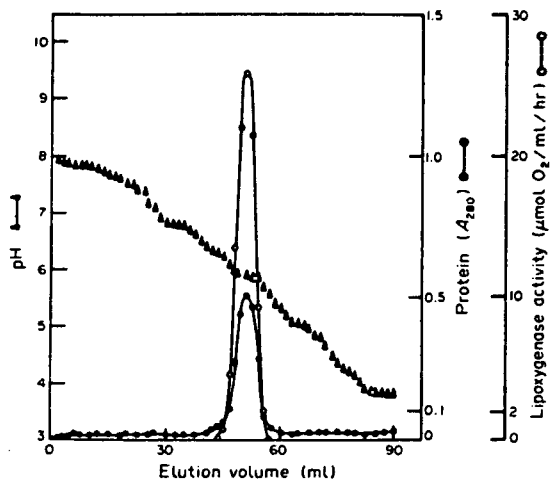

Fig. 3. Isoelectric focussing of purified avocado lipoxygenase. Isoelectric focussing was performed at 4.5° for 3 days. The protein sample was focussed in a pH gradient of 3.5–8.0 at a final ampholyte concentration of 1%. Fractions of 1.5 ml were collected. Protein was determined at 280 nm, activity 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

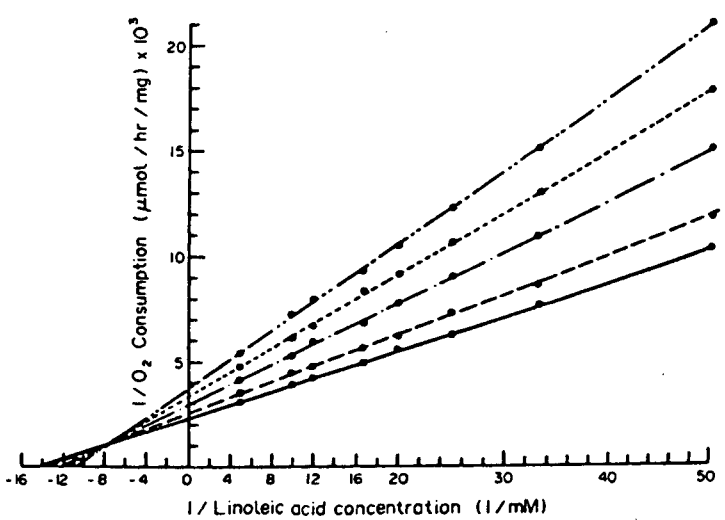

Fig. 4. Reciprocal plot of purified avocado lipoxygenase activity concentration measured by O_2 uptake as a function of linoleic acid only (——), or when supplemented with 4 μg/ml (– – – – –), 10 μg/ml (–·–·–), 20 μg/ml (– – – – –), or 40 μg/ml (–··–··–··) epicatechin.

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	74 000
	Gel electrophoresis	73 600
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 74 000 obtained by gel filtration and by SDS gel electrophoresis is similar to the values found for other lipoxygenases, e.g. 85 000 and 66 000 for horse bean lipoxygenase [22] and 67 000 and 72 000 for pea lipoxygenase [23]. The M_r of avocado lipoxygenase is smaller than that of soybean lipoxygenase, *ca* 100 000, [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 20 000 *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 20 000 *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.

Highly purified avocado lipoxygenase was incubated with various concns of epicatechin {either commercial (Sigma), or an extd and purified preparation from avocado [12]} for several time periods in 20 mM K-Pi buffer, pH 7.0, at 30°. Enzyme activity was monitored after the incubation.

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Effect of Particle Size of Activated Charcoal on Separation of Triton X-100 from Protein, Liver Cytosol, and Lipoxygenase Extracts[1]

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Triton X-100 was almost completely removed from bovine serum albumin solutions, BALB/c mouse liver extracts, and avocado peel lipoxygenase extracts by stirring the samples for 30 min in the presence of 250–350 mesh activated charcoal. The procedure did not remove protein significantly and did not reduce enzyme activity. At higher charcoal particle sizes, the efficiency of Triton adsorption was decreased and protein adsorption was increased. Elevated temperatures enhanced Triton and protein adsorption. Adsorption on activated charcoal of 250–350 mesh is a simple and rapid procedure for Triton removal at a ratio of 0.23 g Triton X-100 per gram of activated charcoal.

KEY WORDS: lipoxygenase; enzyme purification; detergent separation; Triton X-100; activated charcoal.

Triton X-100 is commonly used as the detergent for the emulsification of water-insoluble substrates, for extractions, and for the solubilization and assay of membrane-bound enzymes (1,2). The use of Triton X-100 is limited by its foaming characteristic, inhibition of enzyme activity, low efficiency to solubilize proteins from protein–lipid bonding, and interference with the Folin–Lowry protein assay (3,4). Most methods employed for removal of Triton X-100 from enzyme extracts, such as dialysis (5,8), density gradient centrifugation (6,8), gel filtration (1,2,7,8), and Amberlite XAD-2 or Bio-Beads SM-2 (8,9), are slow, expensive, and complicated. Moreover, these methods can be used only for small volumes, necessitate dilution of the samples, or have low adsorption capacity. Activated charcoal was used previously to adsorb Triton X-100 (8) but was found to be less efficient than XAD-1-7 beads, Amberlite, or Bio-Beads (8). This report shows that the efficiency of Triton X-100 adsorption can be much improved when smaller activated charcoal particles are used.

MATERIALS AND METHODS

Materials

Activated charcoal (250–350, 14–60, 4–14 mesh), bovine serum albumin (BSA),[3] and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Tritium-labeled Triton X-100 [*phenyl*-^{3}H(N)] was obtained from New England Nuclear, U.K.

Methods

Batch procedure for removal of Triton X-100. The Triton X-100 removal was determined in three systems: BSA solutions,

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[3] Abbreviations used: BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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BALB/c mouse liver cytosol extracts, and extracts of avocado peels containing the enzyme lipoxygenase. Mouse liver was homogenized (10% w/v) in Dulbecco's phosphate-buffered saline (Biological Ind., Kibutz Beit HaEmek, Israel), with 0.1% Triton X-100 and used after centrifugation at 8000*g*. Preparation of the lipoxygenase extracts has been described elsewhere (10). In brief, 10 g of avocado peels homogenates in 100 ml 50 mM potassium phosphate, pH 7.2, and various concentrations of Triton X-100 after centrifugation were used. Cytosol and enzyme extracts were stirred at 4°C and BSA solutions at 20°C in the presence of 15 g of activated charcoal (250–350 mesh if not described differently) per 100 ml extract or solution. Duplicate, 10-ml aliquots were removed after various periods of stirring, centrifuged at 35,000*g* for 10 min, and analyzed for Triton X-100 and protein concentration and for lipoxygenase activity.

Triton X-100 was estimated by two methods: (i) spectrophotometrically according to the difference in absorbance at 622 and 687 nm (ΔA 622–687) using ammonium cobalto thiocyanate reagent (11); and (ii) radiochemically: samples containing [^{3}H]Triton X-100 were freeze-dried and counted in Bray's scintillator (14) with a MR 300 liquid scintillation counter and corrected for quenching by external standardization. Lipoxygenase activity in the homogenate was determined by monitoring the uptake of O_2 in the presence of linoleate with a Y.S.I. Model 53 oxygen monitor as described by Grossman and Zakut (12). Protein was estimated by the method of Mather and Tamplin (13).

RESULTS

Adsorption from BSA Solution: Effect of Particle Size and Temperature

The adsorption of Triton X-100 from a BSA solution increased with the decrease of activated charcoal particle size and concomitantly the adsorption of BSA decreased (Fig. 1). During 30 min of stirring at 20°C, 4–14 mesh, 14–60 mesh, and 250–350 mesh charcoal removed, respectively, 30, 46, and 97% of the initial 1% v/v Triton X-100 from the 1% w/v BSA solution. The large activated charcoal particles, 4–14 mesh and 14–60 mesh, removed some BSA from the solution as well (45 and 20%, respectively) while less

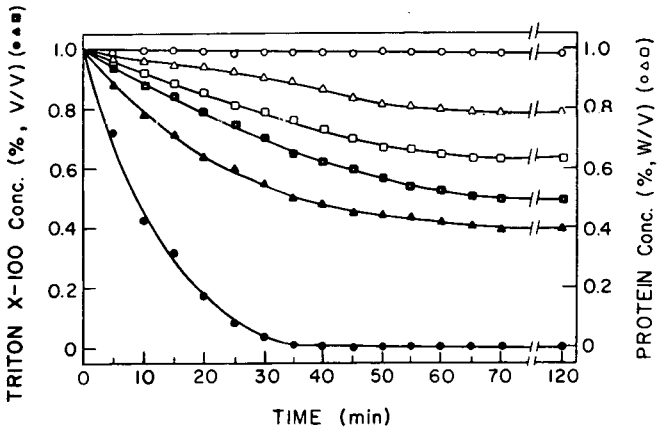

FIG. 1. Effect of activated charcoal particle size (in mesh) on Triton X-100 and protein removal from solution by the batch procedure. Bovine serum albumin solutions 1% (w/v) containing 1% (v/v) Triton X-100 were stirred in the presence of 0.15 g/ml activated charcoal of 4–14 mesh (□,■); 14–60 mesh (△,▲); and 250–350 mesh (○,●) at 20°C for different periods. Triton X-100 (●,▲,■) and protein (○,△,□) remained in solution after adsorption on activated charcoal.

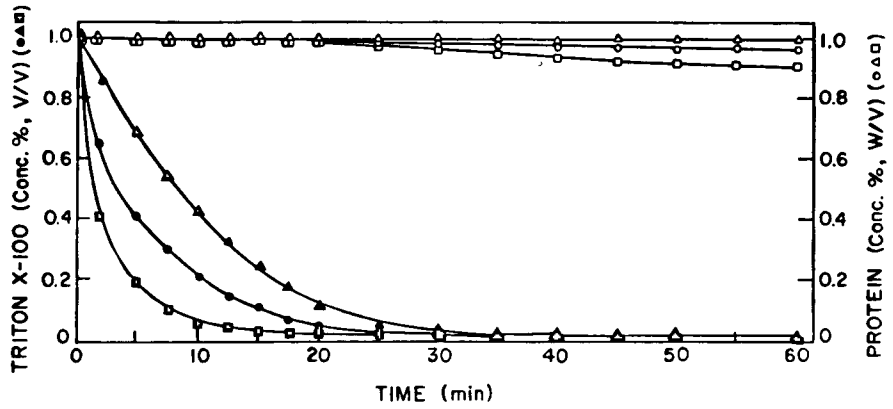

FIG. 2. Effect of temperature on Triton X-100 and bovine serum albumin adsorption by activated charcoal. Activated charcoal (250–350 mesh, 45 g) was added to 300 ml 1% (v/v) Triton X-100 and 1% (w/v) BSA and stirred at: 4°C (▲,△); 30°C (●,○); and 50°C (■,□). Triton X-100 (▲,●,■) and BSA (△,○,□) were determined in periodically removed 10-ml aliquots.

than 3% was removed by the 250–350 mesh activated charcoal.

The initial rate of Triton X-100 removal from BSA solutions with 250–350 mesh activated charcoal increased with temperature (Fig. 2). Then during 10 min, 95% of the Triton was removed from a 1% (v/v) solution at 50°C, as compared with 60% at 5°C. However, after 30 min 98% of the Triton was adsorbed at all temperatures employed. Elevated temperatures resulted also in some increase in BSA adsorption by the activated charcoal, but this was significant only at 50°C and with an incubation period longer than 30 min.

Adsorption from Liver Cytosol

Liver cytosol was homogenated in the presence of the initial concentration of 0.1%

TABLE 1

EFFECT OF ACTIVATED CHARCOAL PARTICLE SIZE (IN MESH) ON TRITON X-100 AND PROTEIN REMOVAL FROM BALB/c MOUSE LIVER EXTRACTS FOR DIFFERENT PERIODS BY THE BATCH PROCEDURE

Time (min)	Triton X-100 concn (% v/v)[a]		Protein concn (mg/ml)	
	4–14 mesh	250–350 mesh	4–14 mesh	250–350 mesh
0	0.082	0.082	0.46	0.46
5	0.085	0.057	0.38	0.45
10	0.073	0.034	0.30	0.44
15	0.066	0.016	0.24	0.43
20	0.058	0.011	0.20	0.43
30	0.050	0.005	0.17	0.43
40	0.047	—	0.17	0.41
60	0.046	—	0.17	0.41
90	0.045	—	0.16	0.41
120	0.045	—	0.16	0.40

[a] The liver was homogenized in 0.1% Triton X-100. The results indicate the remaining Triton X-100 after adsorption in the activated charcoal.

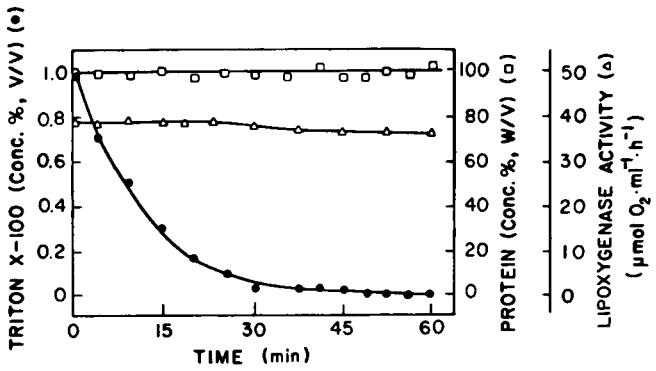

FIG. 3. Rate of removal of Triton X-100 from avocado lipoxygenase solution. A 300-ml sample containing 1% (v/v) Triton X-100 and 1% (w/v) enzymatic protein solution was treated with 45 g activated charcoal by the batch procedure. The concentrations of Triton X-100 (●); and protein (○) and lipoxygenase activity (△) were determined as described in the text. Protein content in the freshly obtained homogenate was 100%.

Triton X-100 corresponding to 0.082% in the supernatant (Table 1). During 40 min of stirring at 4°C, 4–14 mesh and 250–350 mesh removed 43 and 100% of the initial 0.082% (v/v) Triton X-100 from the liver cytosol preparation. The large activated charcoal particles removed 73% of the protein after 40 min of treatment, while 250–350 mesh charcoal only adsorbed 9% of protein.

Adsorption from Lipoxygenase Extracts: Effects of Triton Concentration

Avocado peel was extracted in the presence of 1% Triton X-100; after 30 min of stirring, the supernatant showed 97.5% removal (Fig. 3). Similar removal efficiency was confirmed with [^{3}H]Triton X-100. The concentration of protein in the solution remained unchanged during this period (Fig. 3). The optimal added Triton X-100 concentration for total protein and active lipoxygenase extraction from avocado peels was 2% (v/v) (Table 2). Part of the Triton X-100 was adsorbed to avocado peel debris. At an added initial concentration of 2% (corresponding to 1.36% in the supernatant) and at lower Triton concentration the enzyme seemed to be stable. At higher Triton concentration the total amount of protein extracted remained the same, but lipoxygenase activity decreased and was unstable. When 2% (v/v) of Triton was added during homogenization and 150 mg after centrifugation, the charcoal/Triton ratio in the supernatant was 11 mg μl^{-1} of Triton X-100. This was adequate to remove 95% of the Triton. At higher Triton concentrations, which resulted in lower charcoal/Triton ratios, the residual amount of Triton increased considerably.

DISCUSSION

Charcoal, a nonpolar adsorbant, is particularly effective for adsorbing nonpolar solutes from polar solvents (15), but a low adsorption efficiency was reported in comparison with other adsorbants like Amberlite or Bio-Beads (8,9,15). It is now shown that a decrease in activated charcoal particle size greatly increases adsorption efficiency. Triton X-100 was efficiently removed with 250–350 mesh activated charcoal from protein solutions, liver cytosol extracts, and lipoxygenase homogenates, without significant changes in protein level or lipoxygenase activity.

Triton is apparently adsorbed to activated charcoal as micellar and monomeric Triton (15), and this seems to be related to the adsorbant's surface area. In contrast, large protein molecules were better adsorbed by large activated charcoal particles; hence the selectivity of activated charcoal for Triton versus

TABLE 2

EFFECT OF TRITON X-100 CONCENTRATION ON PROTEIN AND LIPOXYGENASE EXTRACTION FROM AVOCADO PEEL AND ON TRITON REMOVAL DURING 60 MIN INCUBATION AT 4°C WITH 0.15 mg/ml OF 250–350 MESH ACTIVATED CHARCOAL

Added for homogeniz.	Triton X-100 concn (% v/v)		Protein concn (mg/ml)		Lipoxygenase activity μmol O_2 ml^{-1} h^{-1}	
	Initial[a]	Final	Initial[a]	Final	Initial[a]	Final
0.5	0.17	0.003	0.04	0.03	0	0
1.0	0.65	0.005	6.0	5.9	60	58
1.5	1.08	0.010	8.2	8.1	87	85
2.0	1.36	0.010	12.4	12.2	128	126
3.0	2.32	0.050	12.8	12.6	52	32
4.0	3.42	0.025	12.7	12.6	50	31
5.0	4.36	1.010	12.5	12.4	22	11
6.0	5.36	2.040	12.6	12.5	20	8

[a] Found in supernatant after 20,000*g* for 15 min.

protein is increased with decreasing particle size. The rate of Triton binding to activated charcoal increased with temperature; this may be ascribed to an alleviation of the critical micellar concentration and a concomitant increase of monomeric Triton concentration available for adsorption (15).

The optimal Triton X-100 concentration for lipoxygenase solubilization was 2%. At this concentration Triton could be completely removed from the extract with 250–350 mesh activated charcoal without affecting enzyme activity. The described procedure for Triton removal has several advantages. (i) It is rapid, and hence enzymes are exposed only briefly to harmful effects and oxidation. (ii) Triton X-100 removal can be achieved without dilution of the sample, which is important for subsequent preparation for polyacrylamide gel electrophoresis (with and without SDS). (iii) The procedure is particularly suitable for larger volumes of enzyme solution. (iv) The adsorption capacity of activated charcoal is comparable to those of XAD-1-2-4-7, IRC-50, and Bio-Beads, namely 0.23 g Triton X-100 per gram of activated charcoal and 0.12–0.39 g Triton X-100 per gram of the other compounds (8).

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Involvement of epicatechin in the regulation of lipoxygenase activity during activation of quiescent Colletotrichum gloeosporioides infections of ripening avocado fruits.

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Increased avocado lipoxygenase activity from 20 to 40 umole O_2 h^{-1} g^{-1} fresh weight of peel was detectable in unripe fruits during the first hour after harvest, and on ripening fruits 4 to 5 days after harvest. Concomitantly, the concentration of the antifungal compound cis, cis-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene decreased from approx. 1700 to 200 ug g^{-1} fresh weight of peel. The concentration of epicatechin in the peel was inversely correlated with lipoxygenase activity in each case, and decreased significantly when lipoxygenase increased. The amount of lipoxygenase in the crude extract of the fruit peel, which was determined by the ELISA technique, ranged between 10 and 30 ug g^{-1} fresh weight of peel during ripening, without any linear relation with the changes in the enzyme activity.

The results suggest that the differences in lipoxygenase activity are not correlated with changes in the amount of this enzyme, but rather result from changes in the concentrations of its inhibitor, epicatechin.

Abbreviations used in text: ACE, avocado crude extract; ALOX, avocado lipoxygenase; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; LOX, lipoxygenase (EC 1.13.11.12); PBS, phosphate buffered saline; SBLOX, soybean lipoxygenase.

INTRODUCTION

Colletotrichum gloeosporioides Penz. attacks avocado fruits during the growing season, but the infection remains quiescent until the fruit ripens after harvest (2). Prusky et al. (8) isolated *cis,cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, a preformed antifungal compound present in the peel of unripe avocado fruits. It was suggested that this compound is responsible for the quiescency of *C. gloeosporioides* infections in unripe avocado fruit.

Lipoxygenase (linoleate:oxygen oxidoreductase EC 1.13.11.12) was shown to catalyse the oxidation of the antifungal diene in an *in vitro* system (7). The specific activity of LOX in avocado peels rose by 80% during ripening (7). In the same work there was an indication of the possible function of LOX in reducing the concentration of the antifungal diene during fruit ripening, by infiltrating eicosa-tetraynoic acid and x-tocopherol, which are known to inhibit LOX *in vitro*, and which delayed development of disease symptoms in the fruit.

It was further found that LOX activity in the peel of ripening fruits can be regulated by the presence of the endogenous antioxidant epicatechin (9,10). *C. gloeosporioides* symptom appearance after fruit harvest was related to reduction of the epicatechin concentration (10). Furthermore, the decrease of epicatechin to a non-inhibitory concentration was faster in susceptible than in resistant cultivars (9).

The present study was designed to test whether an increase in LOX activity during ripening indeed results from degradation of its endogenous inhibitor epicatechin or from an increase of the amount of enzyme in the fruit. The use of an immunological technique for quantitation of LOX in crude extracts of avocado peel (ACE), enabled us to probe the relationships among LOX activity, LOX amount and epicatechin concentration during fruit ripening.

MATERIALS AND METHODS

Avocado fruits (Persea americana Miller var. drymifolia (Schlect and Chamb) Blake) of the cultivar 'Fuerte' were obtained from an orchard at Ayanot, Israel.

A single spore isolate of C. gloeosporioides from decayed avocado fruits was used for inoculations of fruits in all experiments (7). Darkening of the peel at the inoculation sites in excess of 5 mm diameter eas considered as positive symptom apperance.

Firmness of the avocado fruits was determined by recording the force (kg) required to penetrate the fruit skin and flesh with a 5 mm diam, 4 mm long conic probe (3). The average value from two determinations on each of five fruits is reported.

Extraction and assay of lipoxygenase

Peels (1 to 2 mm thick) of avocado fruits stored at 20°C were extracted at various times after fruit harvest, as described by Prusky et al. (10) with some modifications. Twenty five g of peel was homogenized in 50 ml of ice cold 5mM sodium phosphate buffer, pH 7.2, containing 2% Triton X-100. The extract was filtered through four layers of cheesecloth and centrifuged at 20,000 x g for 15 min. The supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA), kept on ice, and used as an enzyme source.

Lipoxygenase activity was determined by monitoring the uptake of O_2 with a Y.S.I. Biological Oxygen Monitor (Yellow Springs Instrument

Co., Yellow Springs, OH, U.S.A.) in the presence of linoleate at 36°C. Buffered 7.5 mM linoleate solution, containing Tween 20, was prepared as described by Ben-Aziz et al. (1) 2.5 ml was placed into the reaction vial with 0.5 ml of avocado peel supernatant. Oleic acid (Sigma) was used as the control substrate (4). Enzyme activity was expressed as umole O_2 h^{-1} g^{-1} fresh weight.

Preparation of lipoxygenase-specific antibodies

Lipoxygenase from avocado peel, purified approximately 35-fold (4), was used for the immunization of five female Balb/c mice. Five subcutaneous injections of 50 ug enzyme per mouse, emulsified in complete Freund's adjuvant (Disco), were given at 2-week intervals. One week after the last injection, all mice were injected with 5×10^5 NSO myeloma cells, to produce ascitic fluids. The ascites were tested for the presence of anti-enzyme antibodies by ELISA.

Competitive immunoassay for LOX quantitation

The murine ascitic fluids were used as the source of anti-LOX antibodies and the optimal parameters for a solid phase ELISA were determined. A standard competitive immunoassay based on the ELISA was established.

A commercial preparation of soybean lipoxygenase (SBLOX, Sigma grade V) was absorbed by plates (Dynatech) of 96 wells, by adding to each well 50 ul of a 10 ug ml^{-1} SBLOX solution in 50 mM carbonate buffer, pH 9.6, and incubating the plates for 60 min at 37°C. After washing the plates twice with saline, the remaining non-specific binding sites were blocked by adding to each well 100 ul of 2% BSA in

100 mM tris buffer, pH 7.2. After incubation for 60 min at 37°C, the plates were washed four times with saline and immediately used for the immunoassay.

Samples to be assayed were prepared by serial two-fold dilutions of the ACE (designated as "sample free antigen") in 2% BSA-100 mM Tris buffer, pH 7.2, and added to the wells (50 ul per well). Ascitic fluids containing anti-LOX antibodies were diluted (1:200) in 2% BSA-100mM tris buffer, pH 7.2, and added to the wells (50 ul). After incubation for 90 min at 37°C, the plates were washed five times with saline. Fifty ul per well of rabbit antimouse IgG antibodies conjugated to alkaline phosphatase (Sigma, diluted 1:500 in 2% BSA-tris buffer) were added and the plates were incubated for 60 min at 37°C. After washing the plates five times with saline, 50 ul of freshly prepared 1 mg ml^{-1} solution of alkaline phosphatase substrate (Sigma, p-nitrophenyl phosphate, disodium) in diethanolamine buffer, pH 9.8, was added to each well. The enzyme reaction proceeded at 37°C for 30 min and the amount of the coloured product was measured as absorbance at 405 nm in a MicroELISA Auto Reader (Bio-Tek Instruments, EL-310).

For each assay, a standard curve was prepared by use of commercial SBLOX dilutions ("standard free antigen") in PBS, to give a concentration range of 5 to 100 ng per well. The standard curve was plotted for each experiment (Fig. 1a) and used for comparison between assays and as arbitrary units for estimation of LOX content in experimental preparations.

For calculation of LOX quantity in the ACE, a curve of the results obtained for the different dilutions was plotted for each sample (Fig. 1b), and the dilution showing 50% inhibition of binding was defined. The value was multiplied by the dilution factor (from the experimental curve) and the result was expressed as ug LOX g^{-1} fresh weight. All measurements for a given extract were carried out in triplicates. Standard error for the mean of each triplicate did not exceed 9%.

Analysis of epicatechin and antifungal compound

Epicatechin and the antifungal diene in avocado peel were extracted in triplicates and determined by HPLC according to the methods described by Prusky et al. (8,10). Standard error for the mean of each triplicate of the antifungal diene ranged between 3 and 9% in all experiments.

RESULTS

Lipoxygenase amount and activity, antifungal diene and epicatechin in the peel of freshly harvested fruits

During the first hour after harvest, LOX activity in avocado peel increased from 20 umol O_2 h^{-1} g^{-1} fresh weight to twice that value (Fig. 2). Three hours later the activity decreased to that found in just-harvested fruits. Changes in the amounts of LOX in the same extracts, as measured by the ELISA procedure, were not related to its activity.

The concentration of the antifungal diene in the peel decreased during the first hour after harvest from 1700 to 200 ug g^{-1} fresh weight and increased again to about the initial value 3 h later (Fig. 3).

The epicatechin concentration varied between 145 and 155 ug g^{-1} fresh weight during the first 2 h after harvest and increased to 250 ug g^{-1} fresh weight 2 h later; then it decreased again to a low value of approx. 90 ug g^{-1} fresh weight, 6 h after harvest (Fig. 3). Fruit firmness did not change significantly during this period and ranged between 10.2 and 9.8 kg.

Lipoxygenase amount and activity, antifungal diene compound and epicatechin concentration in the peel of ripening avocado fruits.

Lipoxygenase activity varied during the first day after harvest, attaining twice its initial value at 24 h after harvest. The activity

increased further on the fourth day after harvest, when it reached a maximum level. Lipoxygenase activity declined thereafter in completely soft fruits, 6 days after harvest (Fig. 4). The enzyme content, as determined by ELISA, ranged from 10 to 30 ug g^{-1} fresh weight of peel, with no direct relation to the enzyme activity. Epicatechin concentration decreased during the first day after harvest from 167 to 52 ug g^{-1} fresh weight when LOX activity started to increase. On the second day, epicatechin concentration increased to its initial value in the peel of freshly harvested fruits. From the second day on, epicatechin concentration decreased to a minimum of 60 ug g^{-1} fresh weight concomitantly with the increase of LOX activity to its maximum (Fig. 5). The increase in LOX activity during the first 4 days after harvest was accompanied by a continuous decrease of the antifungal compound.

DISCUSSION

It was suggested previously that LOX activity during fruit ripening is regulated by its endogenous inhibitor, epicatechin (10). However, in the present study, concentrations of epicatechin and diene as well as LOX activity were determined at each sampling in samples from the same avocado peel. An inverse relationship between LOX activity and epicatechin concentration was found during the first 24 h after harvest (Fig. 3) and during fruit softening (Fig. 5); LOX activity was high (50 umoles O_2 h^{-1} g^{-1} fresh weight peel) when epicatechin concentration was low (60 ug g^{-1} fresh weight). The amount of LOX, as determined by ELISA, was found to range from 10 to 30 ug g^{-1} fresh weight, exhibiting no direct relation with the enzyme activity (Figs. 2 and 4). These results suggested that the changes in LOX activity did not result from changes in the enzyme amount. This situation resembles that of soybean lipoxygenase, the increase of which in protein content was concomitant with a decrease in its activity (5).

During the first hour after harvest, LOX activity increased rapidly; this was accompanied by a decline in the antifungal diene concentration by ca. 90% compared to unharvested fruit (Fig. 3 and ref. 6). Another decline in diene content occurred during fruit softening, beginning on the third day after harvest, coinciding with the second peak of enzyme activity (Fig. 5). These results lend

further support to the previous hypothesis concerning the possible involvement of LOX in the degradation of the antifungal diene (9).

The present data indicate that the variations in LOX activity, involved in the antifungal diene degradation during avocado fruit ripening, are not due to changes in the enzyme protein content, but rather to regulation of the enzyme activity by one of its endogenic regulators, epicatechin.

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FIGURE CAPTIONS

Fig. 1:a) ELISA readings at 405 nm for binding of soybean lipoxygenase (SBLOX) dilutions to antibodies prepared toward purified avocado lipoxygenase.

b) ELISA readings at 405 nm for binding of avocado peel crude extract (ACE) dilutions to antibodies prepared toward purified avocado lipoxygenase.

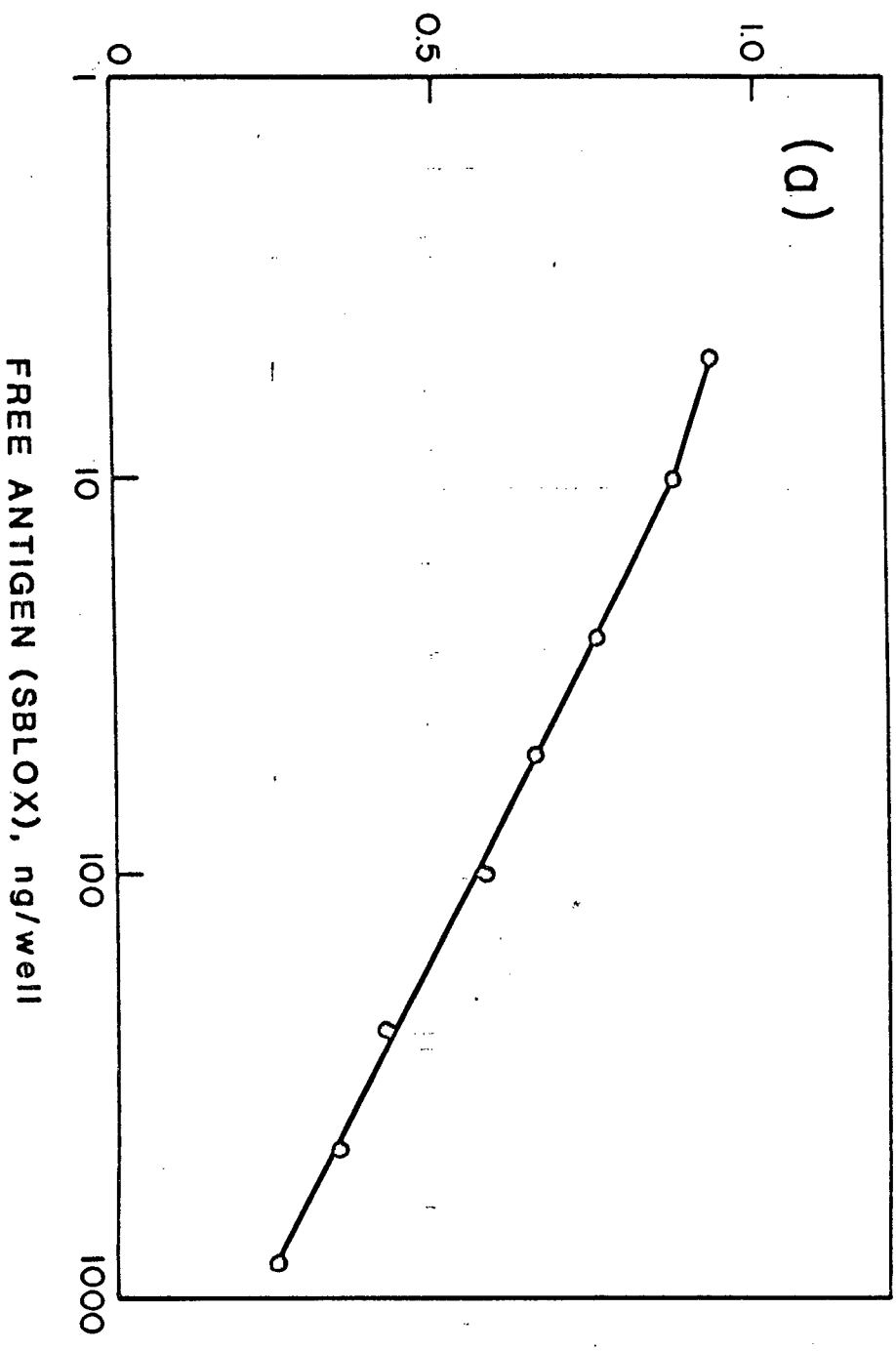
Fig. 2: Lipoxygenase (LOX) activity and amount of lipoxygenase protein in peel of freshly harvested avocado fruits cv. Fuerte stored at 25°C. Peel tissue was homogenized in cold 5 mM sodium phosphate buffer, pH 7.2 containig 2% Triton X-100. LOX activity was determined immediately on the filtered supernatant by oxygen consumption in the presence of linoleate and lipoxygenase protein was determined on the same extract by ELISA as described in materials and methods.

Fig. 3: Lipoxygenase (LOX) activity and antifungal diene and epicatechin concentration in the peel of freshly harvested avocado fruits cv. Fuerte stored at 25°C. The average of three antifungal diene extracts is reported. The standard error of the mean ranged between 3 to 9% in all the experiments.

Fig. 4: Fruit firmness, lipoxygenase (LOX) activity and amount of LOX protein in the peel of avocado fruits cv. Fuerte during storage at 25°C. Peel tisue was homogenized in cold 5 mM sodium phosphate buffer, pH 7.2 containing 2% Triton X-100.

LOX activity was determined immediately on the filtered supernatant by oxygen consumption in the presence of linoleate and lipoxygenase protein was determined on the same extract by ELISA as described in materials and methods. Arrow indicates C. gloeosporioides symptom appearance in ripen fruits after harvest. Darkening of the peel at the inoculation sites in excess of 5 mm diameter was considered as positive symptom apperance.

Fig. 5: Fruit firmness, lipoxygenase (LOX) activity, antifungal diene and epicatechin concentration in the peel of avocado fruits cv. Fuerte stored at 25 ^{o}C after harvest. The average of of three antifungal diene extracts is reported. The standard error of the mean ranged between 3 to 9% in all the experiments. Arrow indicates C. gloeosporioides symptom appearance in ripen fruits after harvest. Darkening of the peel at the inoculation sites in excess of 5 mm diameter was considered as positive symptom apperance.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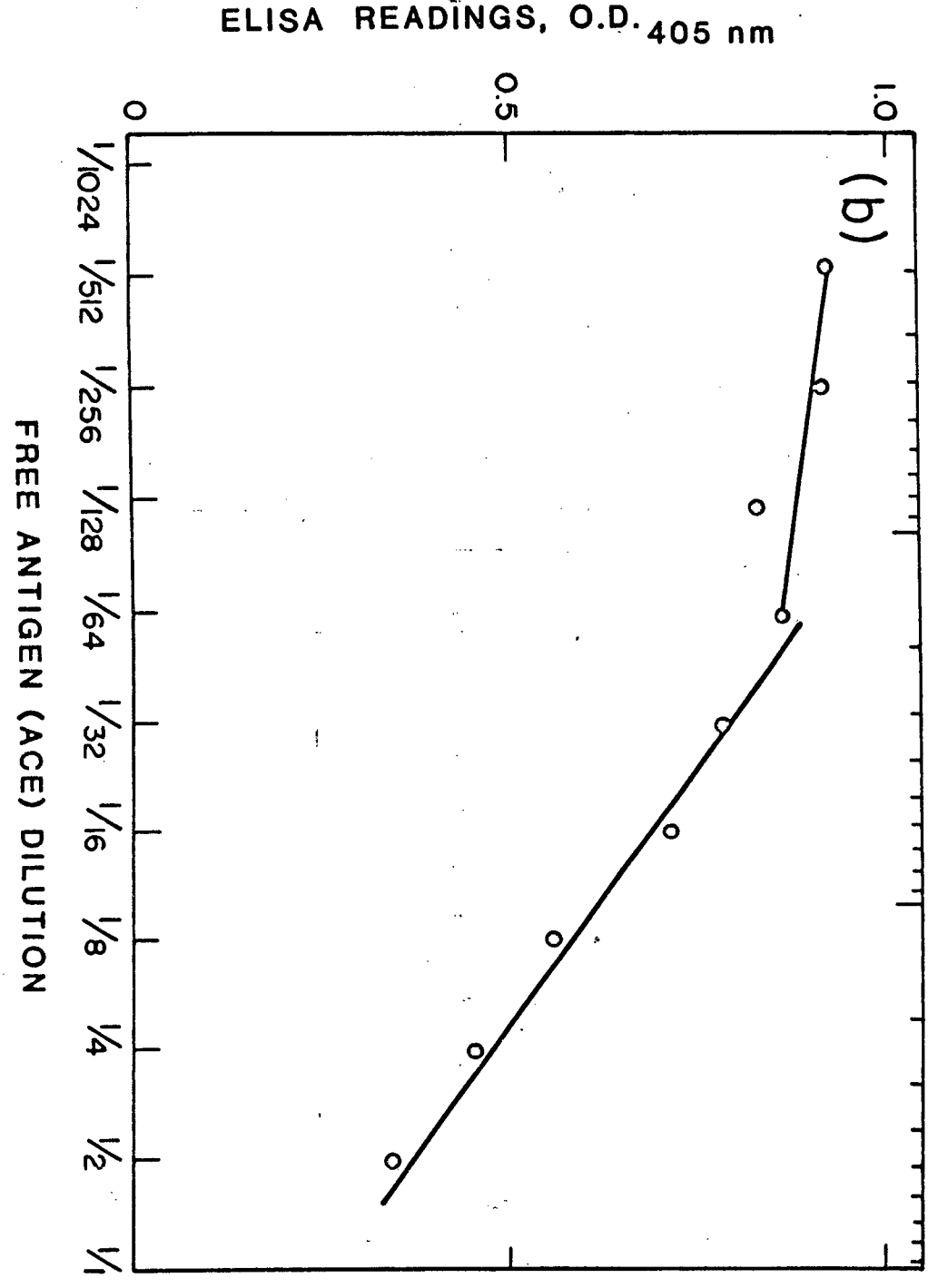
1.0
(a)
FREE ANTIGEN (SBLOX), ng/well
1
10
100
1000

fig 1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1.0
(b)
1/1024
1/512
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1/128
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1/16
1/8
1/4
1/2
1/1
FREE ANTIGEN (ACE) DILUTION

Fig 1

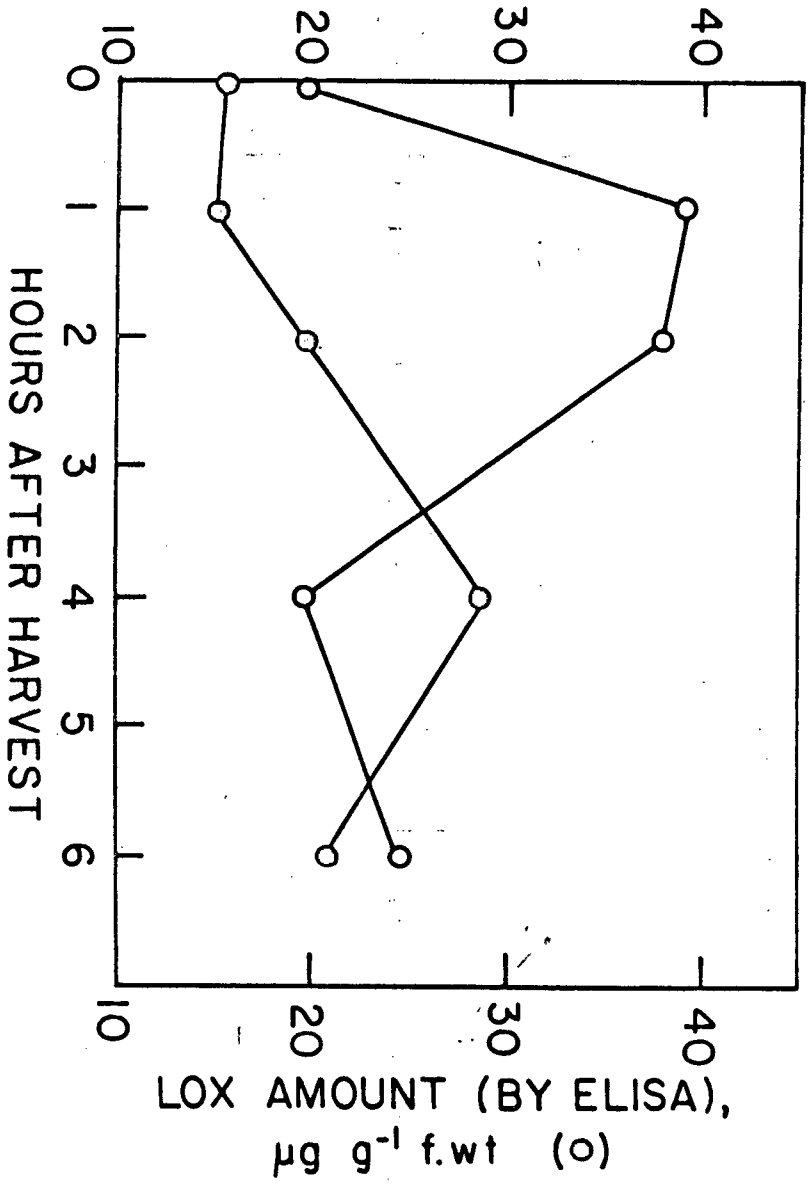


Fig. 2

Fig 3

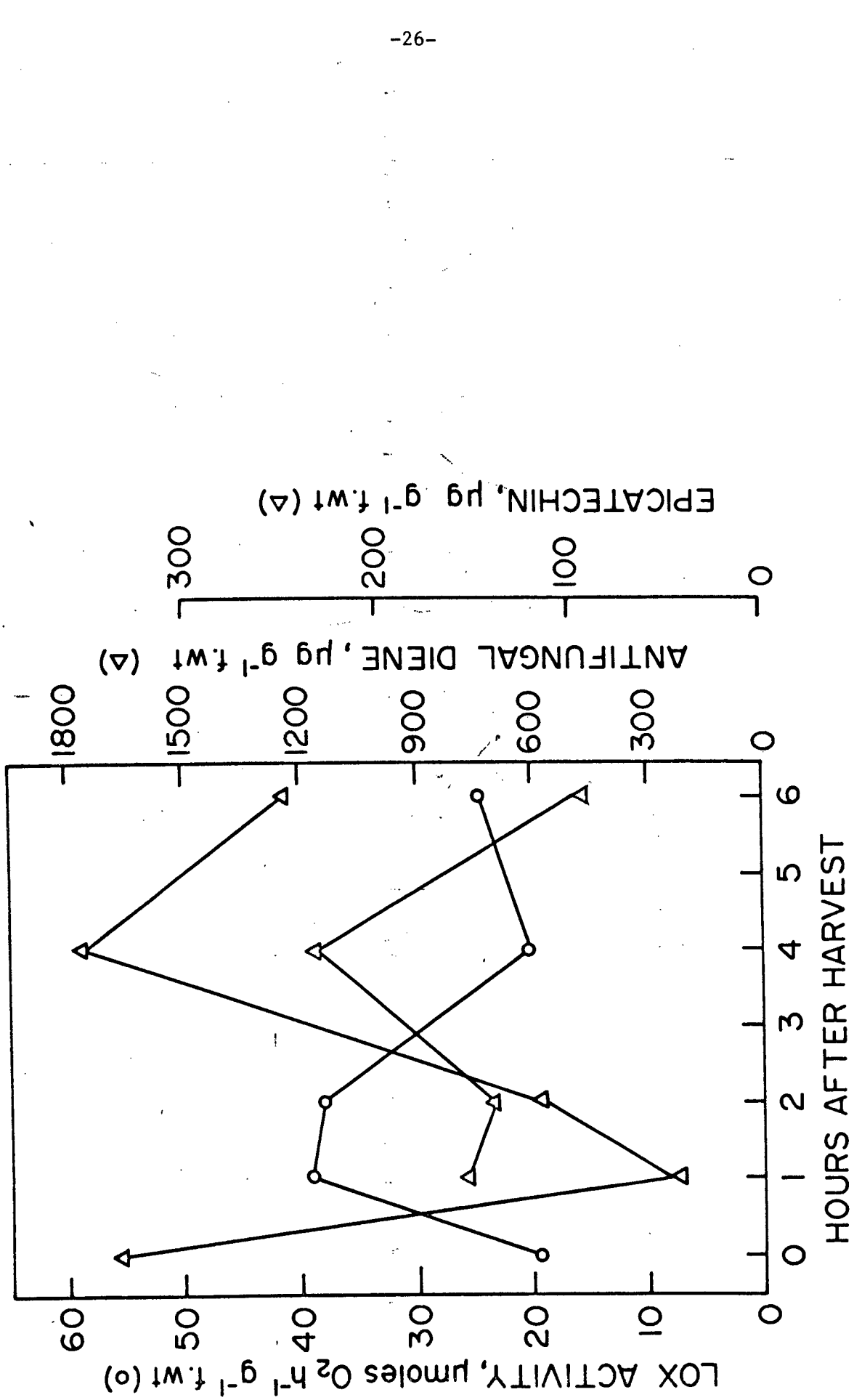
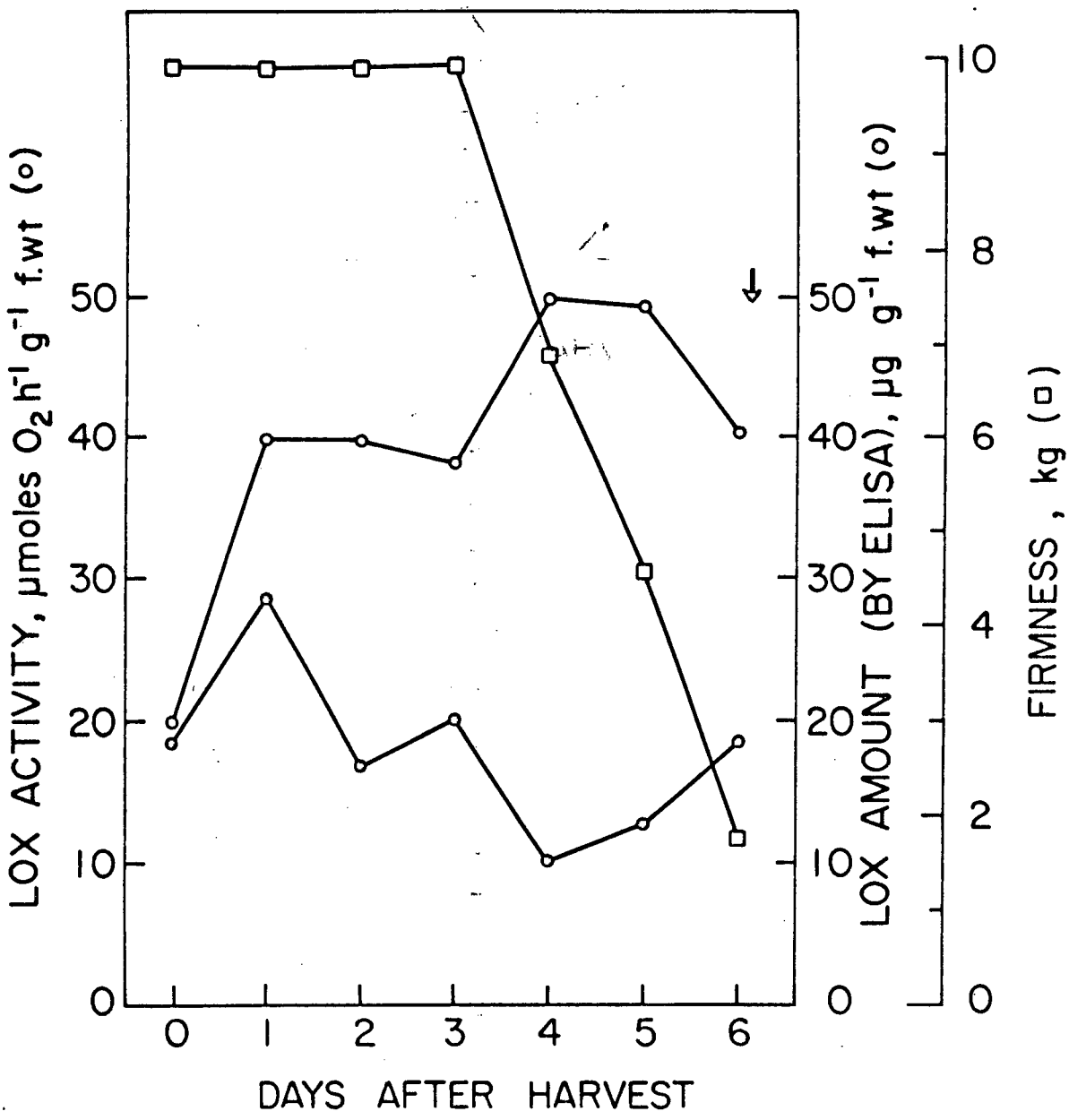
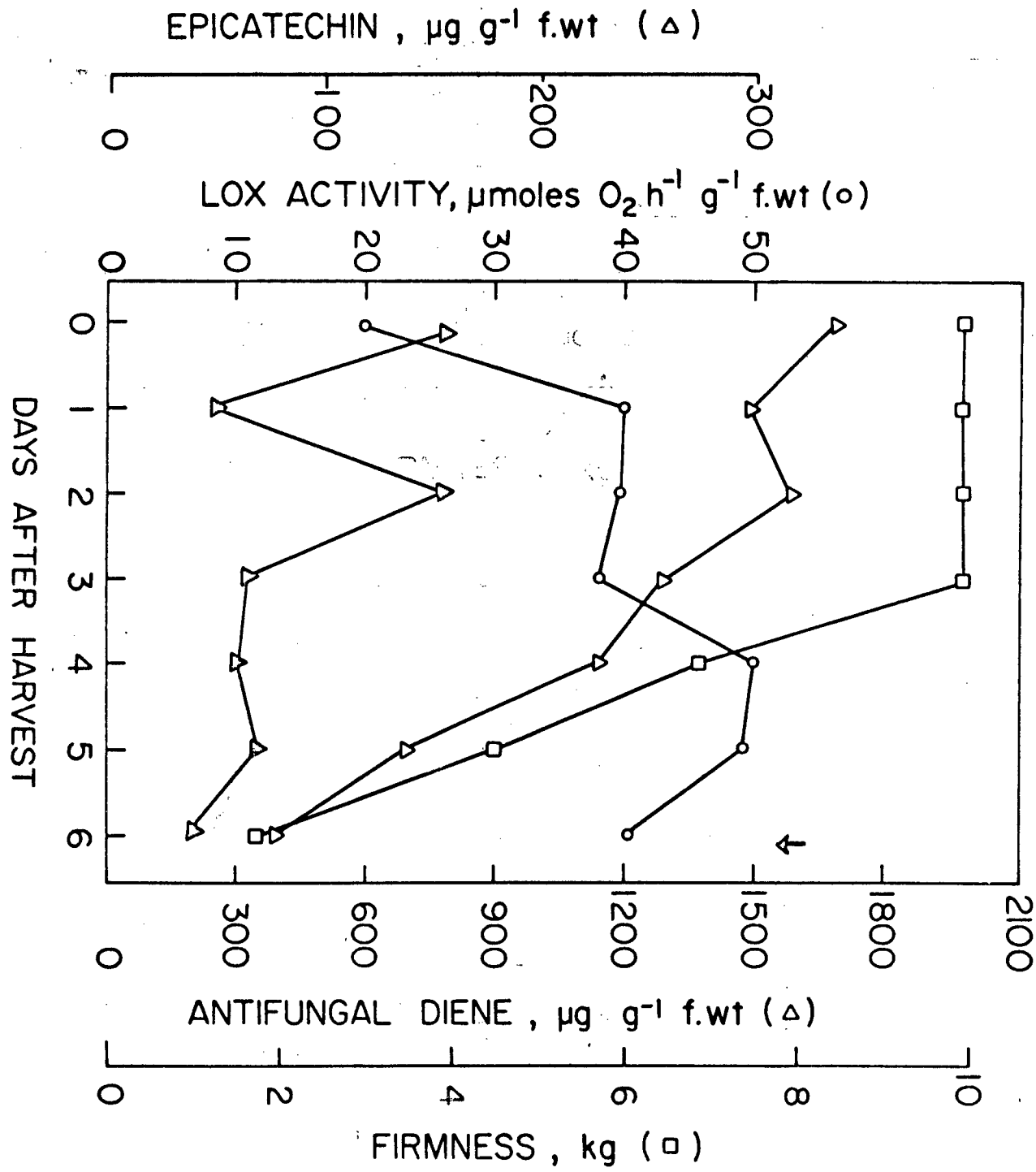

LOX ACTIVITY, μmoles O_2 h^{-1} g^{-1} f.wt (o)
0
10
20
30
40
50
60
HOURS AFTER HARVEST
0
1
2
3
4
5
6
ANTIFUNGAL DIENE, μg g^{-1} f.wt (Δ)
0
300
600
900
1200
1500
1800
EPICATECHIN, μg g^{-1} f.wt (Δ)
0
100
200
300

Fig 4


LOX ACTIVITY, μmoles O_2 h^{-1} g^{-1} f.wt (o)
LOX AMOUNT (BY ELISA), μg g^{-1} f.wt (o)
FIRMNESS, kg (□)
DAYS AFTER HARVEST
0
10
20
30
40
50
0
2
4
6
8
10
0
1
2
3
4
5
6


EPICATECHIN , µg g-1 f.wt (Δ)
0
100
200
300
LOX ACTIVITY, µmoles O2 h-1 g-1 f.wt (o)
0
10
20
30
40
50
DAYS AFTER HARVEST
0
1
2
3
4
5
6
0
300
600
900
1200
1500
1800
2100
ANTIFUNGAL DIENE , µg g-1 f.wt (Δ)
0
2
4
6
8
10
FIRMNESS , kg (□)

3. Involvement of epicatechin in cultivar suceptibility of avocado fruits to *C. gloeosporioides* after harvest.

P118/87C

Agricultural Research Organization, The Volcani Center, Bet Dagan; and Department of Agricultural Botany, The Hebrew University of Jerusalem, Rehovot, Israel

INVOLVEMENT OF EPICATECHIN IN CULTIVAR SUSCEPTIBILITY OF AVOCADO FRUITS TO *Colletotrichum gloeosporioides* AFTER HARVEST[1]

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With 2 figures

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ABSTRACT

Avocado cultivars were defined as susceptible and resistant to Colletotrichum gloeosporioides depending upon the length of the incubation period of the fungus after fruit softening. In the susceptible cultivars Fuerte, Horshim, Vurtz, Rincon and Benik, epicatechin concentration of the peel decreased to 60-130 $\mu g/g^{-1}$ fr. wt. at fruit softening and symptoms appeared on the same or one day later. In the resistant cultivars Hass, Nabal, Netaim and Pinkerton, epicatechin concentration was still 632-1740 $\mu g/g^{-1}$ ft. wt. when fruit softened and symptoms appeared only 4-10 days later. When susceptible Fuerte fruits became soft the concentration of the antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene, had decreased to 120 $\mu g/g^{-1}$ fr. wt. and symptoms appeared. In resistant Hass fruits, the antifungal diene was still 238 $\mu g/g^{-1}$ ft. wt. at fruit softening; and it had further decreased to 159 $\mu g/g^{-1}$ fr. wt. when symptoms appeared, four days later. A modified atmosphere and 0.2 M $CaCl_2$ infiltration both delayed softening of Fuerte fruits; but symptom appearance on these fruits was related to diene decrease and not to fruit softening.

The results are discussed in relation to the hypothesis that the susceptibility of avocado cultivars to postharvest decay by C. gloeosporioides is related to the degradation of the antifungal diene, catalyzed by avocado lipoxygenase, the activity of which is regulated by the decline of its inhibitor epicatechin.

The preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene is apparently responsible for the quiescence of Colletotrichum gloeosporioides Penz. infections in unripe avocado fruits (Prusky et al., 1982). Activation of quiescent infections seems to dependent on the postharvest decrease of the antifungal diene that is catalyzed by endogenous lipoxygenase (Prusky et al., 1983) and regulated by the enzyme's inhibitor, epicatechin (Prusky et al., 1985). Zauberman and Schiffmann-Nadel (1977), investigated factors determining the length of the incubation period of Fusarium sp., another quiescent fungal infection on avocado fruit. They suggested that the incubation period is related to the duration from harvest to fruit ripening (softening); namely the earlier the fruit softens the shorter is the incubation period of infective fungi. Since the concentrations of the antifungal diene decrease in many avocado cultivars simultaneously with fruit softening, it has been difficult to determine whether renewed development of quiescent fungi depend only on the diene decrease or also on fruit softening. The present work was designed to investigate the relative importance of fruit softening and the processes leading to diene decrease in the resistance of avocado cultivars to postharvest development of C. gloeosporioides. It also proposes criteria for classifying avocado cultivars according to their differential postharvest susceptibility.

MATERIALS AND METHODS

General

Avocado fruits of cultivars Fuerte, Vurtz, Rincon, Horshim, Benik, Pinkerton, Hass, Netaim and Nabal were obtained from orchards near Rehovot, Israel. Firmness, an inverse parameter of ripening, was measured by recording the force (kg) required to penetrate the fruit skin and flesh with a conic probe

(5 mm in diameter and 4 mm in length). The average of two determinations on each of five fruits is reported. The fruit was considered soft when firmness reached < 1 kg.

A single spore isolate of C. gloeosporioides fruits was used for inoculation in all experiments (Prusky et al., 1982). This isolate was maintained by inoculation of avocado fruits every 10 days or on Petri dishes with potato dextrose agar incubated at 25°C. Freshly harvested fruits were inoculated by placing a 10 µl drop of a spore suspension (10^6 spores/ml^{-1}) at three different spots along the longitudinal axis of each of five fruits. These fruits were incubated at 20°C in high humidity. Disease expression was defined as the occurence of darkening over an area of 5 mm in diameter. The incubation period was defined as the number of days taken for these symptoms to be observed in 50% of inoculated sites.

Extraction and quantitative analysis of epicatechin and the antifungal compound

Epicatechin and the antifungal diene in avocado peel were extracted according to the methods described by Prusky et al. (1982, 1985) and determined by HPLC. For epicatechin determination, 100 µl aliquots of the 1.0 ml concentrated flash-chromatographed material from the crude extract, obtained from 10 g fr. wt. were chromatographed (Prusky et al., 1985). For diene determination, 100 µl aliquots of the 1.0 ml concentrated biologically active fraction isolated by flash chromatography from an extract of 30 g fr. wt., were chromatographed (Prusky et al., 1982). Epicatechin was monitored with a UV detector at 275 nm and the antifungal diene with a differential refractive index detector. Calculations of concentrations of epicatechin and the diene compound were based on the comparison of the HPLC peak areas of the compounds with those

of standards. The averaging of three separate determinations of each compound is reported.

Fruit Treatments

Treatments affecting fruit ripening were applied to Fuerte fruits. Fruit firmness was determined at different intervals after harvest in uninoculated fruits.

Modified atmosphere treatment.- A modified atmosphere was obtained by storing avocado fruits continuously in closed 2-l jars with a small hole in the lid. The jars were incubated at 20°C and gas samples were taken every hour. A concentration of 8% CO_2 and 13% O_2 was reached after 8 h. These concentrations with a standard deviation of 0.5% was maintained during the whole experiment. Control fruits were kept at the same temperature, under atmosphere conditions.

$CaCl_2$ treatment.- Fruits were completely immersed in 0.2 M $CaCl_2$ or H_2O and infiltrated by reducing the ambient pressure to 150 mm Hg for 2 min. The amount of the solution infiltrated was 0.5 ± 0.2 g per 100 g of fruits. Fruits were then stored at 20°C.

Each experiment affecting fruit ripening was repeated at least twice.

CO_2 and O_2 Determination

CO_2 and O_2 were determined by gas chromatography according to Eaks (1966). The instruments were calibrated for each set of samples by injecting 10 ml of standardized CO_2-nitrogen and O_2-nitrogen, mixtures.

RESULTS

The relation between fruit softening, epicatechin decrease and incubation period of C. gloeosporioides in avocado cultivars.

When the concentration of epicatechin was determined in the peel of nine avocado cultivars during postharvest ripening, it was possible to divide the cultivars into two groups (Table 1). One group included Fuerte, Rincon, Horshim, Vurtz, and Benik, where the epicatechin concentration at softening ranged between 60 and 130 $\mu g/g^{-1}$ fr. wt. In these cultivars symptoms appeared on the same or one day after fruits became soft. These cultivars were considered to be susceptible. The second group of cultivars included Netaim, Hass, Pinkerton and Nabal, where epicatechin concentration at softening ranged between 636 and 1740 $\mu g/g^{-1}$ fr. wt., and symptoms appeared 5-10 days later. These were considered resistant cultivars.

Epicatechin and diene decrease in a susceptible and a resistant cultivars.

The time sequences of epicatechin and diene decrease were determined in the peel of one susceptible (Fuerte) and one resistant (Hass) cultivar (Table 2). Epicatechin concentration in the peel of Fuerte fruits decreased to 12 $\mu g/g^{-1}$ fr. wt. concommitantly with fruit softening. In cv. Hass, 10 days after harvest, when the fruit was completely soft (firmness $\leq$ 1 Kg), the epicatechin concentration was still 456 $\mu g/g^{-1}$ fr. wt. Four days later, epicatechin decreased to 15 $\mu g/g^{-1}$ fr. wt.

Diene concentration in cv. Fuerte was 1420 $\mu g/g^{-1}$ fr. wt. one day after harvest and then decreased to 120 $\mu g/g^{-1}$ fr. wt. on the 6th day, when symptoms appeared. In the peel of cv. Hass fruits, the diene concentration was 620 $\mu g/g^{-1}$ fr. wt. 2 days after harvest and it decreased to 238 $\mu g/g^{-1}$ fr. wt. in

soft fruits, 10 days after harvest. Symptoms appeared 4 days later, when the diene concentration had decreased to 159 $\mu g/g^{-1}$ fr. wt.

The fruit peel of all the resistant cultivars is thicker than that of the susceptible ones. It was determined whether this affects the incubation period of C. gloeosporioides. Fruits from resistant and susceptible cultivars, Hass and Fuerte respectively, were inoculated when the diene concentration had decreased to subfungitoxic levels. Under these conditions the incubation period was 2 days in both groups of cultivars.

Effect of modified atmosphere and $CaCl_2$ on fruit firmness, diene concentration and symptom appearance.

Untreated Fuerte fruits softened nine days after harvest, together with disease symptom appearance. The diene concentration in these fruits was 116 $\mu g/g^{-1}$ fr. wt. (Fig. 1). At the same time modified atmosphere-treated fruits were still firm and symptomless, and their diene concentration was 3415 $\mu g/g^{-1}$ fr. wt. After three additional days, symptoms of C. gloeosporioides appeared on modified-atmosphere treated fruits, and the diene concentration had decreased to 160 $\mu g/g^{-1}$ fr. wt., but fruit firmness was still 8.5 kg.

Treatments with $CaCl_2$ prevented softening of Fuerte fruit. Their firmness was 4.8 and 0.3 kg respectively in treated and untreated fruits on the 8th day after harvest when C. gloeosporioides symptoms appeared in treated as well as untreated fruits (Fig. 2). Upon symptom appearance, diene concentration was 5 $\mu g/g^{-1}$ fr. wt. in calcium-treated and 75 $\mu g/g^{-1}$ fr. wt. in untreated fruits, where symptoms had appeared.

DISCUSSION

Binyamini and Schiffmann-Nadel (1972) and Schiffman-Nadel et al. (1970) reported that the avocado pathogens C. gloeosporioides and Diplodia natalensis remained quiescent in firm fruits, and that rot development started concommitantly with or after fruit softening. The present comparison of nine avocado cultivars showed, that the renewal of quiescent C. gloeosporioides development after harvest and appearance of decay were not necessarily related to fruit softening. Cultivars that are generally considered as susceptible showed symptom development coincident with fruit softening, but those that are considered resistant showed decay symptoms only 4-10 days after fruit softening.

Development of C. gloeosporioides decay on ripening avocado fruits was usually found (Prusky et al., 1982) to occur when the preformed antifungal diene had decreased to less than 200 $\mu g/g^{-1}$ fr. wt. Oxydation of the diene seems to be catalyzed by lipoxygenase (Prusky et al., 1983) and regulated by the decrease of the enzyme's endogenous inhibitor, epicatechin, during ripening (Prusky et al., 1985). The present results show that epicatechin concentrations in cultivars of the susceptible group, decreased to levels of 60-130 $\mu g/g^{-1}$ fr. wt. when the fruits softened, while in resistant cultivars, in the same experiment, epicatechin concentrations ranged still between 636 and 1740 $\mu g/g^{-1}$ fr. wt. at the time of softening. In a separate experiment with the susceptible and resistant cultivars Fuerte and Hass, diene concentration was determined in addition to epicatechin. At softening, the diene concentration in the resistant cultivar was also higher than in the susceptible one, and above the subfungitoxic level (Table 2). These results seem to indicate that the process leading to increased lipoxygenase activity and diene decrease, are responsible for symptom appearance

(Prusky et al., 1982) and not fruit softening. Further support for the lack of a relationship between fruit softening and disease appearance comes from results of experiments with modified atmosphere and with calcium treatments of Fuerte fruits. Both treatments delayed fruit softening, as already reported by Spalding and Reeder (1975) and Tingwa and Young (1974). However, this delay did not prevent symptom appearance; the latter concurred with diene decrease to subfungitoxic levels.

In addition to delayed diene disappearance, resistant avocado cultivars differ from sensitive ones in their thicker peel. Thickness of avocado fruit peel cultivars ranges between less than 1 and over 2 mm (Hodgson, 1950). Thin-peeled cultivars are considered those with less than 1 mm thick, while thick-peeled cultivars over 1 mm thickness. When fruits of thick- and thin-peeled cultivars were inoculated with C. gloeosporioides, after diene had decreased to subfungitoxic levels, the incubation period was identical. This indicated that diene concentration, and not peel thickness per se, is responsible for differential postharvest resistance of avocado cultivars.

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Table 1. Comparison of fruit softening, epicatechin concentration and incubation period of Colletotrichum gloeosporioides in several avocado cultivars stored at 20°C after harvest.

Cultivar	Days to softening*	Epicatechin at softening** ($\mu g/g^{-1}$ fr. wt.)	Incubation period (days)
Fuerte	11	60	11
Rincon	10	70	10
Vurtz	15	100	15
Benik	16	110	16
Horshim	9	130	10
Netaim	10	636	15
Hass	16	1500	24
Pinkerton	8	1740	16
Nabal	14	822	24

*Softening was considered firmness ≤ 1 kg.

**The average of three separate determinations is reported.

Table 2. Fruit firmness, epicatechin and antifungal diene concentrations and symptom appearance of Colletotrichum gloeosporioides on the peel of cvs. Fuerte and Hass avocado fruits stored at 20°C.

Days after harvest	Fuerte				Hass			
	Firmness (kg)	Epicatechin* $\mu g/g^{-1}$ fr. wt.	Diene	Symptom appearance	Firmness (kg)	Epicatechin* $\mu g/g^{-1}$ fr. wt.	Diene	Symptom appearance
1	10.7	433	1420	-				
2					12.3	519	620	-
4	5.2	344	540	-				
6	0.5	12	120	+				
7					8.5	591	321	-
10					1.0	456	238	-
14					0.5	15	159	+

*The average of three separate determinations of each compound is reported.

Figures

Fig. 1. Effect of modified atmosphere on fruit firmness (▽--▽), concentration of the antifungal diene (□,▨) and symptom appearance (↓) of Colletotrichum gloeosporioides in Fuerte fruits stored at 20°C. The modified atmosphere was obtained by storing freshly harvested fruits in jars with a small outlet, in which the atmosphere contained 9% CO_2 and 13% O_2 (▼,▨). Untreated fruits ( ) were stored at normal atmosphere.

Fig. 2. Effect on infiltration with 0.2 M $CaCl_2$ on fruit firmness (▽--▼), concentration of the antifungal diene (□,▨) and symptom appearance (↓) of Colletotrichum gloeosporioides in Fuerte fruits stored at 20°C. Fruits were infiltrated with $CaCl_2$ (▼,) or water (▽,▨) 24 h after harvest.

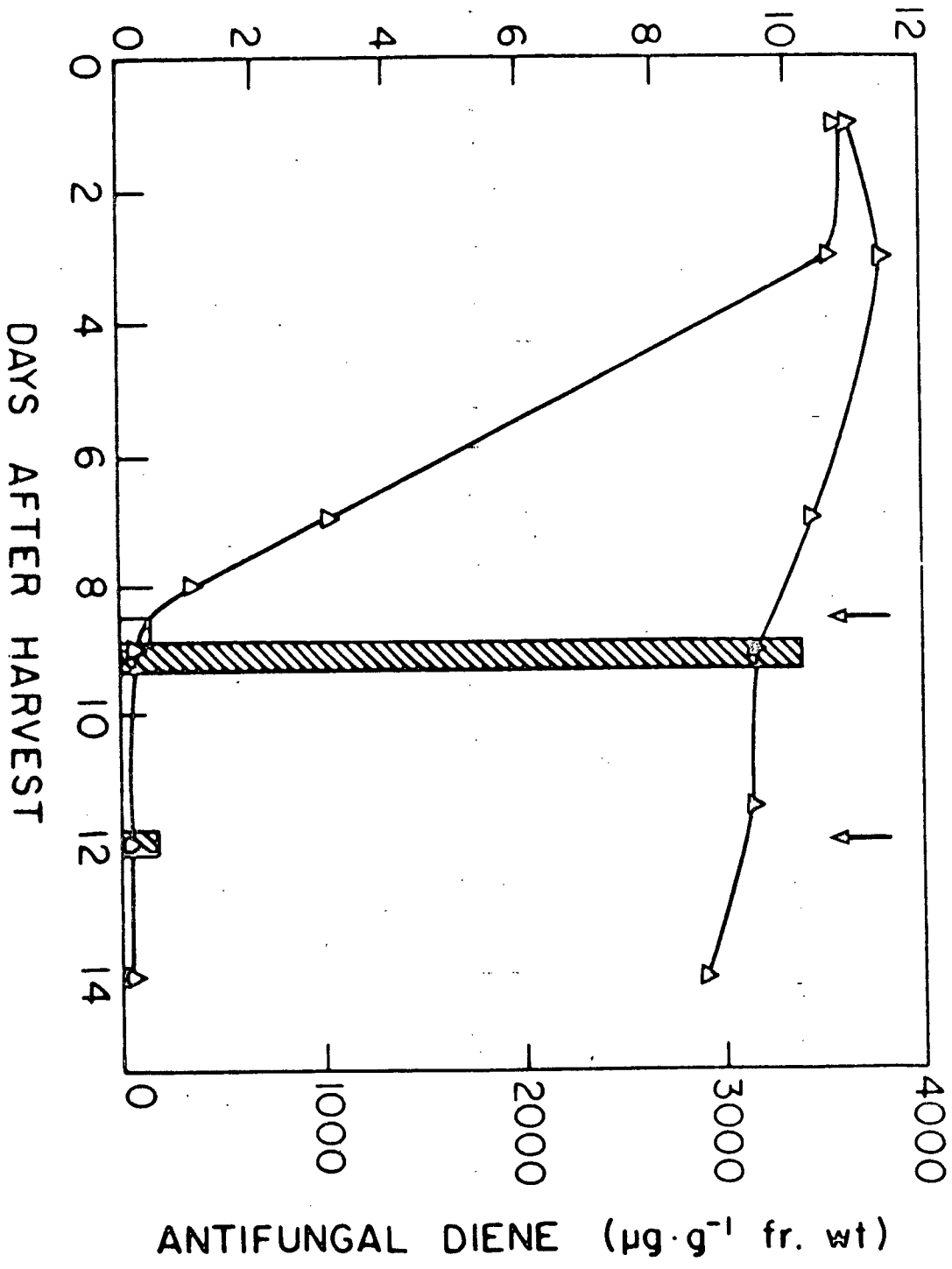
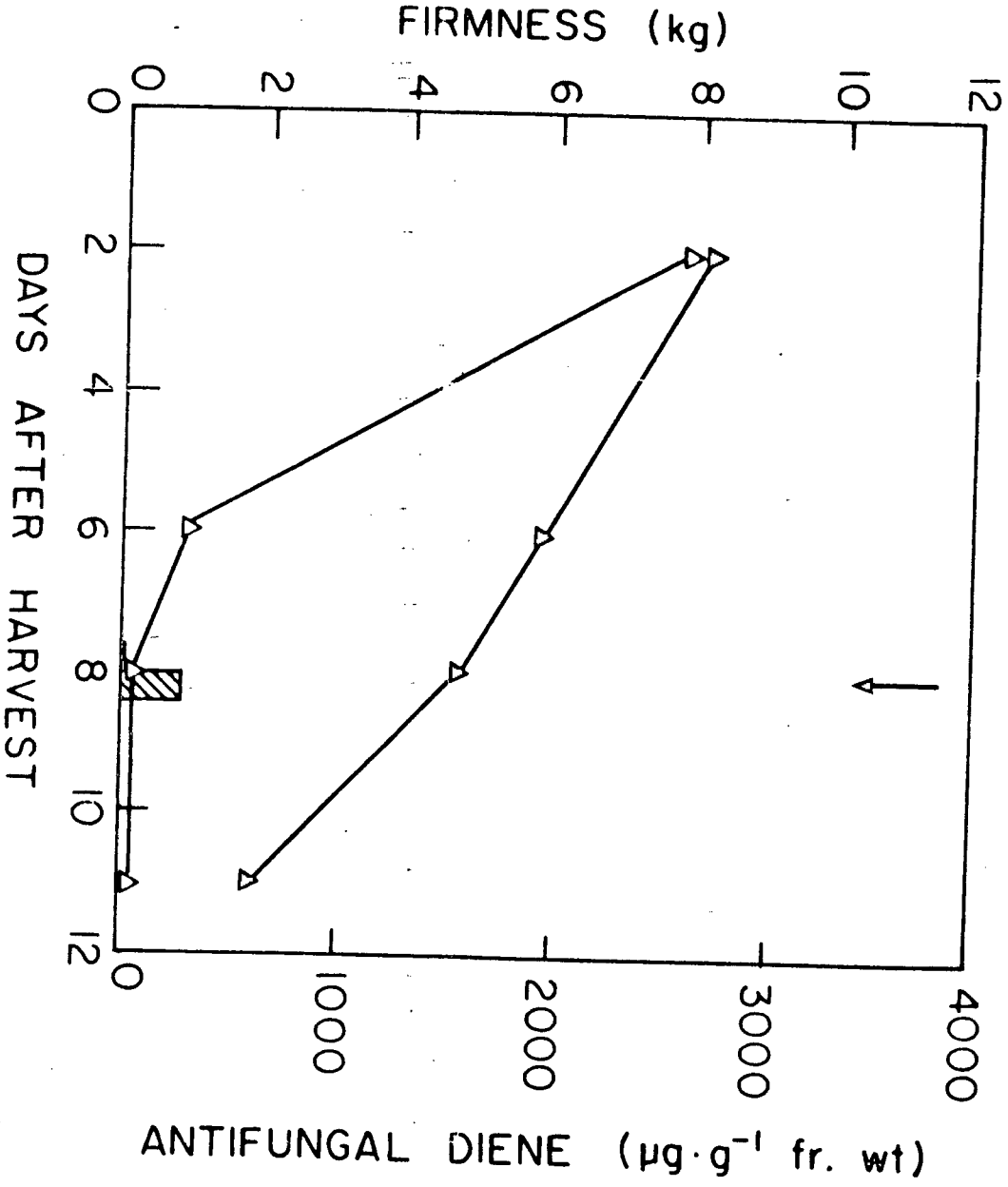

FIRMNESS (kg)
0
2
4
6
8
10
12
DAYS AFTER HARVEST
0
2
4
6
8
10
12
14
0
1000
2000
3000
4000
ANTIFUNGAL DIENE (μg·g⁻¹ fr. wt)

Fig 1.


FIRMNESS (kg)
0
2
4
6
8
10
12
DAYS AFTER HARVEST
0
2
4
6
8
10
12
0
1000
2000
3000
4000
ANTIFUNGAL DIENE (μg·g⁻¹ fr. wt)

4. The use of antioxidants to delay the onset of anthracnose and stem end decay in avocado fruits after harvest.

The Use of Antioxidants to Delay the Onset of Anthracnose and Stem End Decay in Avocado Fruits After Harvest

DOV PRUSKY, Department of Fruit and Vegetable Storage, ARO, The Volcani Center, Bet Dagan 50250, Israel

ABSTRACT

Prusky, D. 1988. The use of antioxidants to delay the onset of anthracnose and stem end decay in avocado fruits after harvest. Plant Disease 72:381-384.

Infiltration of avocado fruits (cultivar Fuerte) with 0.1 mM solutions of an antioxidant (butylated hydroxy toluene [BHT], α-tocopherol, *tert*-butylhydroquinone [TBHQ], epicatechin, or 0.01% gum guaiac) delayed the appearance of anthracnose symptoms for 1–2 days. A mixture of 0.1 mM BHT and either 0.5% ascorbic or 0.1% citric acid enhanced the activity of BHT alone and delayed the appearance of anthracnose symptoms for a further day without affecting fungal growth in vitro. Butylated hydroxy anisole and propyl gallate (0.1 mM) had no effect on disease expression. Treatments of fruits with 0.1 mM epicatechin or a mixture of 0.1 mM BHT and 0.5% ascorbic acid delayed the disappearance of endogenous epicatechin and the antifungal diene, and delayed disease expression; infiltration with 1 mM TBHQ, however, enhanced the disappearance and disease expression. In semicommercial trials, stem end rot in cultivars Ettinger and Fuerte was also delayed for 1–4 days as a result of dip treatment in a mixture of 0.1 mM BHT and either 0.5% ascorbic or 0.1% citric acid.

Additional keywords: antifungal compounds, quiescent infections, postharvest diseases, postharvest treatments

Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., and stem end rot, caused by *Diplodia natalensis* Pole-Evans, are important fruit-rot diseases of avocado in the U.S.A., Israel, and Australia (8,12,17,18). *C. gloeosporioides* infects avocado peel (2) and *D. natalensis* infects the fruit pedicel (1) during the growing season, but the infections remain quiescent until fruit ripen after harvest. Under commercial conditions, decay development can usually be delayed either by storage at low temperature (5–6 C) (13) or by fungicide treatment (7). The latter is applied as preharvest orchard sprays or, more efficiently, as postharvest dip treatments (7).

Prusky et al (10) isolated a preformed antifungal compound from the peel of unripe avocado fruits and identified it as *cis,cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene (Fig. 1). This compound was shown to be involved in the mechanism for quiescence of *C. gloeosporioides* infections in unripe avocado (10). The antifungal diene also inhibited elongation of germinating *D. natalensis* hyphae with an ED_{50} similar to that of *C. gloeosporioides* (480 μg/ml). It was suggested that lipoxygenase catalyzes the oxidation of the antifungal diene during fruit ripening, resulting in its decrease to subfungitoxic concentrations and, therefore, in its decrease in activation of fungal infections (Fig. 2) (9). Infiltration of fruits with α-tocopherol (BDH), an inhibitor of lipoxygenase activity, inhibited the decrease of the antifungal diene and also the development of anthracnose lesions (9). It was concluded that lipoxygenase activity in the peel of ripening fruits could be regulated by concentration of the natural antioxidant epicatechin (Fig. 2) (11). Reduction in concentration of epicatechin in the peel after harvest was always found to be associated with a decrease in diene concentration and development of anthracnose lesions (*unpublished*).

This contribution reports on exogenous postharvest treatments that inhibited lipoxygenase activity and delayed the activation of quiescent infections and development of anthracnose and stem end decay.

MATERIALS AND METHODS

Avocado fruits (cultivars Ettinger and Fuerte) were obtained from an orchard near Rehovot, Israel. A single-spore isolate of *C. gloeosporioides* from decayed avocado fruits was used in all experiments. Cultures of this strain were maintained either on avocado fruits or on potato-dextrose agar (PDA) plates (10). Fruits were inoculated with a 10-μl drop of a spore suspension (10^6 spores/ml) placed at four different positions along both sides of the longitudinal axis of each of 20 freshly harvested fruits and then incubated at 20 C. Fruits were examined daily for disease symptoms using a binocular microscope. Peel darkening exceeding 1 mm in diameter was considered positive for disease expression. The effects of the treatments were expressed as days from inoculation to disease expression. Standard deviation was calculated for 80 infection points per treatment.

Effect of antioxidants on *C. gloeosporioides* decay development. Twenty-four hours after inoculation, opposite halves of the same fruits were infiltrated with either antioxidant or solvent solutions (9). Infiltration was carried out by immersing the appropriate half of the fruit and reducing the pressure to 100 mm Hg for 60 sec. The amount of solution absorbed by each half fruit was 0.20 ± 0.08 g per 100 g of fruit. Fruits were infiltrated with α-tocopherol, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), gum guaiac, *tert*-butylhydroquinone (TBHQ), and epicatechin in 0.1% ethanol, 0.1% dimethyl sulfoxide (DMSO), and 0.01% Triton X-100. The same detergent-ethanol mixture, without antioxidant, served as a control. In some experiments, ascorbic or citric acid at 0.5% and 0.1%, respectively, were added to BHT as

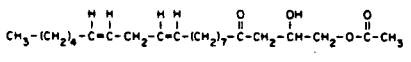


Fig. 1. Structural formula of the antifungal diene from avocado.

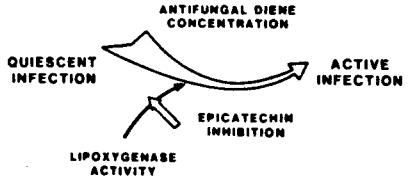


Fig. 2. Sequence of reactions leading to active infections of *Colletotrichum gloeosporioides* in ripening avocado: lipoxygenase-catalyzed decrease in diene concentration facilitates activation of quiescent infection. Epicatechin inhibits lipoxygenase activity.

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synergists of antioxidant activity (14). Experiments were repeated at least twice.

Effect of antioxidants on *C. gloeosporioides* growth in vitro. Antioxidants dissolved in 95% ethanol were amended into PDA. Antioxidant-amended and unamended plates were inoculated with 5-mm-diameter plugs of fungal mycelium sampled from a 7-day-old culture. The diameter of growth developed at 20 C and was checked daily. Presence of ethanol at a concentration of 1% in the media did not affect *C. gloeosporioides* growth.

Extraction and quantitative analysis of the antifungal diene and epicatechin. The antifungal diene was extracted from avocado peels and quantified as described by Prusky et al (10). The lipoxygenase inhibitor, epicatechin, was extracted in 5-mM sodium phosphate buffer (pH 7.2), partially purified by flash chromatography, and quantified by HPLC as described by Prusky et al (9).

Effect of antioxidant and fungicide dip treatment on development of *D. natalensis* decay in semicommercial trials. In these experiments, fruits were not artificially inoculated. To assure a high incidence of stem end rot, avocado cultivars Fuerte and Ettinger were picked early during the harvesting season from orchards known for high incidence of the disease (12,13). The fungicide imidazole (Prochloraz, 45 EC) 1-*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]carbamoylimidazole (7,12) was compared with antioxidant treatments for control of stem end rot. Twenty-four hours after harvest, fruits were dipped for 30 sec in 900 μg/ml prochloraz or in aqueous antioxidant solutions at 0.1 mM BHT and 0.5% ascorbic acid, and 0.1 mM BHT and 0.1% citric acid. After treatment, fruits were air-dried and stored at 20 C or stored at 2 C for 10 days and then transferred to 20 C. Symptoms of Diplodia stem end rot exceeding 1 cm deep in the fruit flesh were considered positive for disease expression. Each treatment was replicated six times with 20 fruits in each replicate. Experiments were conducted twice in each of two harvesting seasons.

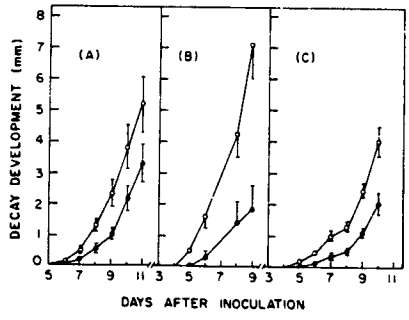

Fig. 3. Effect of antioxidants on decay development of *Colletotrichum gloeosporioides* in avocado fruits stored at 20 C. One-half of the whole fruit was infiltrated 24 hr after inoculation of freshly harvested fruits with **(A)** butylated hydroxy toluene, **(B)** α-tocopherol at 0.1 mM, and **(C)** gum guaiac at 0.01% in 0.01% Triton X-100, 0.1% DMSO, and 0.1% ethanol (●). Control halves (○) were infiltrated with the solvent alone. Decay development is expressed as the diameter of peel darkening at the inoculated site. Darkening of the peel exceeding an area of 1 mm in diameter was considered as symptom expression of disease. Standard deviation is provided in bracket.

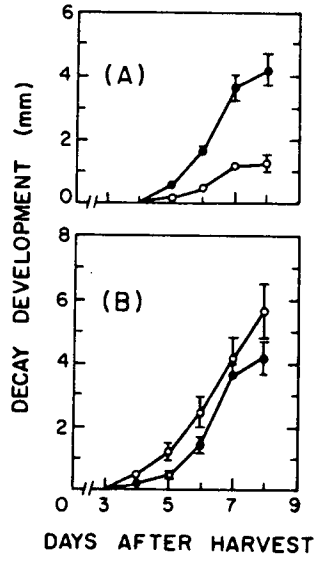

Fig. 4. Effect of *tert*-butylhydroquinone on decay development of *Colletotrichum gloeosporioides* in avocado fruits stored at 20 C. Freshly harvested fruits were inoculated. Twenty four hours later, one half of the whole fruit was infiltrated either with the solvent (controls) consisting of 0.01% Triton X-100, 0.1% DMSO, and 0.1% ethanol (○), or with solvent also containing TBHQ (●) at concentrations of **(A)** 1 mM or **(B)** 0.1 mM. Decay development is expressed as the diameter of peel darkening at the inoculated site. Darkening of the peel exceeding an area of 1 mm in diameter was considered symptom expression of disease. Standard deviation is provided in bracket.

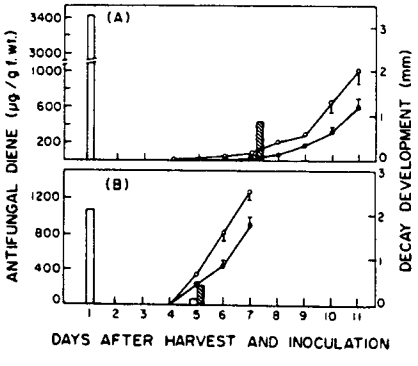

Fig. 5. Effect of epicatechin on the antifungal diene concentration and decay development by *Colletotrichum gloeosporioides* in avocado fruits stored at 20 C. Epicatechin (0.1 mM), in 0.01% Triton X-100, 0.1% DMSO, and 0.1% ethanol (●), was infiltrated into fruit halves 24 hr after inoculation of freshly harvested fruits. Control halves were infiltrated with the solvent alone (○). Concentration of the antifungal diene in crude extracts of peel treated with epicatechin (hatched bar) or the solvent alone (open bar). Two different experiments were carried out **(A)** in the beginning of the harvest season and **(B)** 3 mo later. Darkening of the peel exceeding an area of 1 mm in diameter was considered symptom expression of disease. Standard deviation is provided in bracket.

RESULTS

Effect of antioxidants on *C. gloeosporioides* decay development. Infiltration of avocado fruits with 0.1 mM BHT, 0.1 mM α-tocopherol, or 0.01% gum guaiac delayed decay development by *C. gloeosporioides* on the treated side of the fruits (Fig. 3) compared with the control side. Butylated hydroxy toluene delayed disease expression by 1 day, and α-tocopherol and gum guaiac delayed it by 2 days. Inhibition of lesion expansion was observed until 9–11 days after harvest in the antioxidant-treated versus control halves of the fruits. Treatments with 0.1 mM BHA or 0.1 mM propyl gallate were ineffective (results not presented). The effect of TBHQ differed with concentration. *Tert*-butylhydroquinone at 0.1 mM delayed disease expression by 1 day (Fig. 4B), but treatment with 1 mM TBHQ significantly hastened disease expression by 1 day (Fig. 4A). Effects of treatments lasted for at least 8 days after harvest.

The addition of either 0.1% citric or 0.5% ascorbic acid enhanced the activity of BHT. In both treatments disease expression was delayed by 1 day. Eight days after inoculation, the lesion diameter on the BHT-ascorbic acid- and BHT-citric acid-treated sides was 1.0 ± 0.2 mm and 0.7 ± 0.3 mm, respectively; in the BHT controls, the lesion averaged 1.8 ± 0.3 mm. Neither 0.1% citric nor 0.5% ascorbic acid affected decay development significantly. Eight days after inoculation, lesion diameter averaged 1.5 ± 0.3 mm on the control side and 1.7 ± 0.5 mm on the ascorbic acid-infiltrated side. On fruits treated with citric acid, the lesion averaged 2.2 ± 0.3 mm on the control side compared with 1.8 ± 0.3 mm on the treated side.

C. gloeosporioides growth was not affected by mixtures of 0.1 mM BHT with either 0.1% citric or 0.5% ascorbic acid added to PDA. After 7 days at 20 C, the diameter of 5-mm fungus circles had grown to 5.8 ± 0.1 mm in the antioxidant-amended and unamended plates.

Effect of antioxidants on epicatechin and diene concentration and on decay development of *C. gloeosporioides*. *Effect of epicatechin treatments on diene concentration and decay development.* Two experiments were carried out during one harvesting season. At the beginning of the season, the concentration of the antifungal diene in the fruit peel was 3,450 μg/g fresh wt 1 day after harvest (Fig. 5A). Infiltration with 0.1 mM

epicatechin delayed the decrease of the antifungal diene. Seven days after harvest, diene concentration was 460 μg/g fresh wt on the epicatechin-treated side and 60 μg/g fresh wt on the control side. Concentrations of endogenous epicatechin on the epicatechin-treated and control sides were 674 and 451 μg/g fresh wt, respectively, 2 days after treatment. Four days after treatment, epicatechin concentrations were similar in both sides. In the second experiment, 3 mo later, the initial concentration of the diene was 1,100 μg/g fresh wt. Five days later, concentrations of the diene on the epicatechin-treated and control sides of the fruit were 250 and 90 μg/g fresh wt, respectively (Fig. 5B). Epicatechin treatment delayed expression of disease in both experiments by 1 day.

Effect of BHT-ascorbic acid treatment on epicatechin and diene concentration. Infiltration of avocado fruits with a mixture of 0.1 mM BHT and 0.5% ascorbic acid delayed the decrease of endogenous epicatechin concentration that occurs after harvest (Table 1). In the peel on the antioxidant-mixture-treated side, the concentration of epicatechin was higher throughout the experiment. The antioxidant treatment also delayed the diene decrease that occurred in the peel on the control side following the fifth day after harvest. On the seventh day, diene concentration on the control side was about 25% of that on the treated side. Nine days after harvest, disease was expressed on the control side, whereas on the treated side it was expressed on the tenth day.

Effect of TBHQ treatment on epicatechin and diene concentration. Infiltration with 1 mM TBHQ enhanced disease expression by 1 day (Fig. 4) and it also enhanced the decrease of endogenous epicatechin and antifungal diene concentration of the fruit (Table 2). Concentration of epicatechin on the control side had decreased to 288 μg/g fresh wt on the eighth day after harvest, whereas on the treated side it was already 62 μg/g fresh wt. Diene concentration in the TBHQ-treated side of the fruit was lower throughout the experiment. On the eighth day after harvest, it was 520 μg/g fresh wt on the treated side and 2,050 μg/g fresh wt on the control side. On the twelfth day after harvest, disease expression occurred on the TBHQ-treated side, whereas on the control side it occurred on the thirteenth day.

Effect of antioxidants and fungicide dip treatment on development of *D. natalensis* decay in semicommercial trials. Dip treatment of Ettinger fruits in a mixture of BHT and either 0.1% citric or 0.5% ascorbic acid delayed symptoms of stem end rot by about 4 days (Fig. 6). After the decay appeared, it increased more rapidly in fruits dipped in BHT and ascorbic acid than in the mixture of BHT and citric acid. Dip treatments of Fuerte fruits (Fig. 7) on a mixture of BHT and citric acid delayed disease expression by 2 days compared with controls. The mixture of BHT and ascorbic acid and the commercial fungicide prochloraz at 900 μg/ml delayed disease expression by only 1 day.

DISCUSSION

The resistance of unripe avocado fruits to attack by postharvest pathogens was associated with the preformed antifungal compound called diene (10). Previous results (9,11) led to the belief that diene decrease was a result of increased lipoxygenase activity. Activity of this enzyme was regulated by the decrease in concentration of the flavan 3-ol inhibitor, epicatechin (11). In the present work, compounds that inhibited lipid peroxidation activity of lipoxygenase (4,16) were tested as possible inhibitors of diene peroxidation. They delayed the development of anthracnose and stem end rot. Infiltration treatments of avocado with α-tocopherol, BHT, TBHQ at 0.1 mM, and 0.01% gum guaiac delayed the expression of anthracnose symptoms by 1–2 days (Figs. 1 and 2).

Treatments with mixtures of 0.1 mM BHT and either 0.5% ascorbic or 0.1% citric acid delayed the expression of disease symptoms by 1 day more than BHT alone. These mixtures did not inhibit the fungus in culture, and their effect must have been on plant metabolism. Plant lipoxygenases are strongly inhibited by some polyphenols

Table 1. Effect of infiltration of avocado fruits (cultivar Fuerte) with a mixture of 0.1 mM butylated hydroxy toluene (BHT) and 0.5% ascorbic acid on the concentration of epicatechin and antifungal diene, and on decay development of *Colletotrichum gloeosporioides* at 20 C

Days after harvest	Epicatechin[a] (μg/g fresh wt)		Diene[a] (μg/g fresh wt)		Decay development (mm)	
	Control	BHT + ascorbic acid[b]	Control	BHT + ascorbic acid	Control	BHT + ascorbic acid
5	913	1,173	1,490	1,500		
6	1,431	1,817	500	1,480		
7	516	786	120	500		
9	12	47			1.9[c]	1.0 *
10					3.3	1.8 *

[a] Samples of partially purified extracts were chromatographed on reverse phase HPLC columns and peak heights were compared with those of standards.
[b] Fruits were infiltrated with an aqueous solution of 0.01% Triton X-100, 0.1% dimethyl sulfoxide, and 0.1% ethanol. The same solution, without the antioxidant, was infiltrated into control fruits.
[c] Fruits were inoculated by placing 10-μl drops of a spore suspension (10^6 spores/ml) at four different positions along both sides on each of 20 freshly harvested fruits. Decay development was measured as darkening of the peel. Expression of disease was recorded when peel darkening exceeded an area of 1 mm in diameter. * = Means for treated versus control were different at $P = 0.05$.

Table 2. Effect of infiltration of avocado fruits (cultivar Fuerte) with 1 mM *tert*-butylhydroquinone (TBHQ) on the concentration of epicatechin and diene, and on decay development by *Colletotrichum gloeosporioides* at 20 C

Days after harvest	Epicatechin[a] (μg/g fresh wt)		Diene[a] (μg/g fresh wt)		Decay development (mm)	
	Control	TBHQ[b]	Control	TBHQ	Control	TBHQ
1	1,235		720			
4	572	233	910	560		
6	639	273	1,940	510		
8	288	62	2,050	520		
11	28	13	830	440		
12					0.7[c]	1.0 *
13					1.0	2.0 *
14					1.6	3.6 *

[a] Samples of partially purified extracts were chromatographed on reverse-phase HPLC columns and peak heights were compared with those of standards.
[b] Fruits were infiltrated with an aqueous solution of TBHQ in 0.01% Triton X-100, 0.1% dimethyl sulfoxide, and 0.1% ethanol. The same solution, without the antioxidant, was infiltrated into control fruits.
[c] Fruits were inoculated by placing 10-μl drops of a spore suspension (10^6 spores/ml) at four different positions along both sides on each of 20 freshly harvested fruits. Decay development was measured as darkening of the peel. Expression of disease was recorded when peel darkening exceeded an area of 1 mm in diameter. * = Means for treated versus control were different at $P = 0.05$.

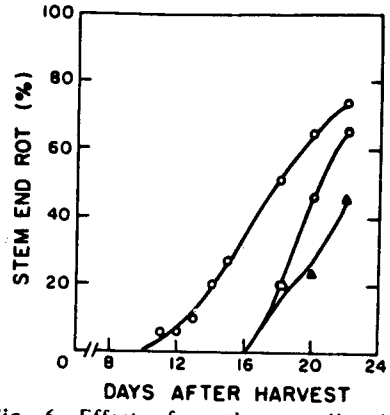

Fig. 6. Effect of postharvest dips in antioxidants on the incidence of *Diplodia natalensis* stem end rot on stored avocado fruit (cultivar Ettinger). Fruits were dipped in a mixture of 0.1 mM BHT and 0.5% ascorbic acid (o) or 0.1 mM BHT and 0.1% citric acid (▲). Controls (●) were dipped in water only. Dipped fruits were stored at 2 C for 10 days and then transferred to 20 C. *D. natalensis* penetration into fruit flesh to a depth of 1 cm was considered rot development.

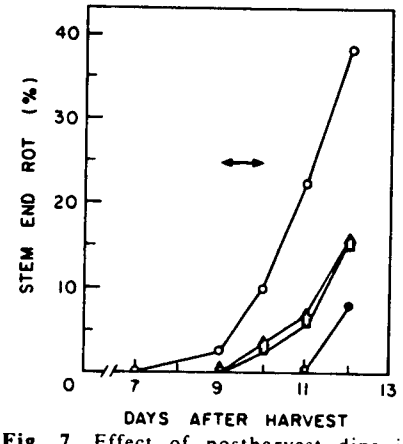

Fig. 7. Effect of postharvest dips in antioxidants and fungicide on the natural incidence of *Diplodia natalensis* stem end rot on stored avocado fruits (cultivar Fuerte). Fruits were dipped in a mixture of 0.1 mM BHT and 0.5% ascorbic acid (□), 0.1 mM BHT and 0.1% citric acid (•), or 900 $\mu g/ml^{-1}$ prochloraz (▲). Control fruits (o) were dipped in water. Fruits were stored at 20 C throughout the experiment. *Diplodia* penetration into fruit flesh to a depth of 1 cm was considered disease expression. Horizontal arrow indicates fruit were completely softened.

(6,15). The inhibition, which is often competitive, is the result of the removal of free radicals required for the reaction (3). When functioning as antioxidants, phenols constitute a trap for free radicals, resulting in their own oxidation to a very stable product (3), preventing epicatechin oxidation and inhibiting lipoxygenase increase and diene decrease. A similar effect was observed with an exogenous treatment of epicatechin, which prevented endogenous epicatechin decrease and delayed the diene decrease.

Tert-butylhydroquinone (0.1 mM) delayed disease expression by 1 day but at 1 mM, epicatechin and antifungal diene decrease and decay development were enhanced (Table 2). Antioxidants at certain concentrations act as prooxidants (5), indicating the importance of optimization of treatments because different concentrations may have opposite effects. This indicates that the regulation of decay development, initiated by the decrease of epicatechin and then of the diene, is a process that might be affected in either way.

The mixture of BHT and ascorbic or citric acid delayed the beginning of stem end rot development in semicommercial experiments by 1–4 days. A mixture of BHT and citric acid was more effective than the commercial fungicide prochloraz used in Israel for this purpose (11).

Treatments of avocado fruits with antioxidants delay the conversion of quiescent infections of *C. gloeosporioides* and *D. natalensis* into active infections and disease. This delay seems to depend on prevention of the decline of the natural mechanisms of resistance.

ACKNOWLEDGMENTS

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F. DESCRIPTION OF COOPERATION.

The cooperation between the Department of Storage of Agricultural Products, ARO and the Department of Agricultural Botany, Hebrew University was very active during the whole project. The work of the Department of Plant Pathology, University of California, Riverside, was tremendously significant for the identification of the inhibitor of avocado lipoxygenase and a new antifungal compound from avocado still not reported. A continuos exchange of ideas and results occured during the whole project between the US and Israeli laboratories . Dr. Keen visited the Volcani to revise current work and to present a talk before the Bat Sheva de Rotchild, Host-Parasite interaction Conference. Dr. Prusky visited twice the Department of Plant Pathology, where he was involved in the purification of the lipoxygenase-inhibitor. It can be summarized that each lab had his significant contribution needed for the success of the project, while all the data was concentrated by the PI in the Department of Storage of Agricultural Products.

G. RESEARCH ACHIEVMENTS.

The research achieved all the original aims of the project. The regulation of the mechanisms of fruit resistance in harvested avocado fruitswas suggested and based on this results a commercial treatment was proposed. The development of this idea will continue in the next years, where several experiments in packing houses will be carried on.

H. LIST OF PUBLICATIONS

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