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ENZYME ACTIVITIES AND POLYPHENOLS RELATED TO MESOCARP DISCOLOURATION OF AVOCADO FRUIT

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Key Word Index—*Persea americana*; Lauraceae; avocado; polyphenol oxidase; peroxidase; phenylalanine ammonia-lyase.

Abstract—Avocado fruit showing severe symptoms of the mesocarp discolouration disorder exhibited significantly higher extractable activities of soluble polyphenol oxidase and peroxidase, as well as higher levels of total phenols, hydroxycinnamic acids and proanthocyanidins, when compared to healthy fruit. However, L-phenylalanine ammonia-lyase activity was very variable, and no significant differences were observed between healthy and affected fruit. Extraction of healthy, but not severely affected, fruit in the presence of 0.1% SDS resulted in increased polyphenol oxidase activity reflecting the release of bound and/or latent enzyme. Qualitative differences between healthy and affected fruit included different patterns of polyphenol oxidase multiple forms and different polyphenol profiles. The pattern of polyphenol oxidase multiple forms from SDS-extracted healthy fruit was similar to that from mildly affected fruit not extracted with detergent.

INTRODUCTION

Mesocarp discolouration of avocado fruit is a post-harvest disorder in which healthy fruit rapidly develop a grey-brown discolouration on the surface of the pulp upon cutting the fruit in half and exposing the cut surfaces to the atmosphere [1–3]. When severe, this disorder affects the whole pulp, and should not be confused with pulpspot, which is a browning reaction limited to the region around cut vascular tissue. Similar symptoms to mesocarp discolouration were attributed to chilling injury [1–3] caused by the storage of avocado fruit over extended periods at low temperatures. Recently, however, it was shown [4] that these symptoms could be reproduced by 'suffocating' the fruit, and were probably a result of the accumulation of CO_2 and lack of O_2 [5].

The Fuerte cultivar of avocado appears to be more susceptible to browning disorders than most other cultivars. Kahn and co-workers [6–8] have shown that the fruit of cultivar Fuerte are highly susceptible to browning, and exhibit higher polyphenol oxidase (PPO, formerly EC 1.10.3.1, now EC 1.14.18.1) activities than other cultivars. The relationships between browning of cut fruit tissues, levels of endogenous phenolic substrates and enzyme activities have been investigated in a number of studies [6, 7, 9–12], although little work seems to have been done specifically on avocado mesocarp discolouration, in particular in fruit which have undergone commercial storage and long-distance transport. In the present paper we investigate the relationships between PPO and phenolic levels in healthy and affected avocado fruit imported from South Africa to the United Kingdom in April–June 1983.

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RESULTS AND DISCUSSION

Inspection of avocado fruit, cv Fuerte, imported to Covent Garden Market, London, by boat from South Africa during April–June 1983 indicated that the earliest batches delivered showed a high incidence (approximately 35%) of fruit with severe mesocarp discolouration. Later fruit showed a much lower incidence (approximately 8%) of the disorder, most of the fruit being completely healthy or showing mild pulpspot symptoms.

Enzyme activities and levels of phenolic compounds

A comparison of the specific activities of the enzymes L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), PPO and peroxidase (PO, EC 1.11.1.7) in healthy, mildly mesocarp discoloured and severely discoloured fruit (Table 1) indicated that: (a) PAL activity varied greatly from fruit to fruit; although the mean activity in the samples appeared to decrease with increasing severity of symptoms, this was not statistically significant. (b) PPO activity was approximately 7-fold higher in severely affected as compared to healthy fruit, and differences between the three classes of fruit were statistically significant; and (c) PO activity was significantly higher in affected than healthy fruit, although there was no significant difference in activity between mildly and severely affected cases.

Levels of total phenols and proanthocyanidins showed statistically significant (approximately 5-fold) increases in affected as compared to healthy fruit (Table 1).

PPO activity in avocado is found in soluble, bound, and latent forms [13–15], the bound enzyme being released, and the latent form activated, by extraction of tissues in buffers containing 0.1% SDS [14]. A comparison of PPO activity in healthy and severely affected fruit indicated that SDS-extraction resulted in greatly increased PPO activities in healthy fruit, but had no effect on the specific

Table 1. Enzyme activities and levels of phenolic compounds in healthy and mesocarp discolouration affected avocado fruit

Symptom	PAL activity (μkat/kg protein)	PPO activity (ΔOD_{420}/min per mg protein)	PO activity (ΔOD_{420}/min per mg protein)	Total phenols (μg equiv. catechol/g fr. wt)	Proanthocyanidins (μmol equiv. grape leucocyanidin/g fr. wt)
Healthy	11.20 ± 6.33(8)*	3.1 ± 1.70(8)	0.17 ± 0.04(8)	96 ± 22(8)	0.55 ± 0.14(8)
Mild mesocarp discolouration	9.96 ± 12.27(16)	14.1 ± 2.70(8)	0.44 ± 0.12(8)	358 ± 60(8)	1.09 ± 0.32(8)
Severe mesocarp discolouration	4.46 ± 4.13(16)	21.4 ± 4.86(8)	0.52 ± 0.09(8)	442 ± 76(8)	2.26 ± 0.56(8)
Statistical significance					
Healthy compared to mild	NS	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$
Mild compared to severe	NS	$P < 0.01$	NS	$P < 0.005$	$P < 0.005$

Values are given ± s.d. for the number of samples analysed.
*Figures in parentheses are numbers of individual fruit assayed.

Table 2. Comparison of soluble and total PPO activities in healthy and mesocarp discolouration affected avocado fruit

	PPO activity (ΔOD_{420}/min per mg protein)		Total protein (mg/g tissue)	
Symptom	Soluble	Soluble + latent + bound	Extraction minus SDS	Extraction plus SDS
Healthy	3.1 ± 1.70(8)*	16.3 ± 4.98†(8)	7.81 ± 0.56‡(8)	6.68 ± 0.73(8)
Severe mesocarp discolouration	21.4 ± 4.86(8)	21.9 ± 4.79†(8)	4.91 ± 0.93‡(8)	5.76 ± 0.75(8)

For measurement of total enzyme activities (soluble + latent + bound), extraction buffer contained 0.1 % SDS. Values are given ± s.d. for the number of samples analysed.
*Figures in parentheses are numbers of individual fruit assayed.
†Values for healthy fruit are significantly lower than for severely affected fruit, $P < 0.02$.
‡Values minus SDS are not significantly different from the corresponding plus SDS values.

activity of the enzyme extracted from severely affected fruit (Table 2). These results represent genuine changes in enzyme activities, as SDS did not significantly increase total extractable protein in either case. It must therefore be concluded that one major biochemical symptom of mesocarp discolouration is the release of bound PPO and/or the activation of latent PPO in the pulp.

Qualitative analysis of PPO multiple forms

Avocado PPO is known to exist in a variety of forms. These have been studied in healthy fruit of cv Fuerte, where they were separated by either polyacrylamide gel electrophoresis, yielding six active bands when stained with either DOPA, caffeic acid, chlorogenic acid or 4-methyl catechol [16]; ion-exchange chromatography on DEAE-cellulose, yielding two major peaks active against 4-methyl catechol [16]; or gel-filtration on Sephadex G-100, yielding two peaks active against 4-methyl catechol [16]. Thin-layer gel-filtration of avocado extracts on Sephadex G-150 yielded five fractions of approximate MWs 14 000, 28 000, 56 000, 112 000 and > 400 000 [17].

In order to investigate whether mesocarp discolouration was associated with qualitative as well as quantitative differences in PPO, extracts from healthy, mildly affected and severely affected fruit were subjected to gel-filtration on Sephacryl S-300 (Fig. 1). Healthy fruit contained one major form of the enzyme of approximate MW 141 000 (form III), a minor form of MW 43 500 (form VI) and traces of a high MW form (MW > 333 000). There was no evidence of forms of MW 28 000 and 14 000, as shown previously by thin-layer gel-filtration [17]. Extracts from mildly affected fruit gave a very different elution profile, with three major forms of MW > 400 000 (form I), 230 000 (form II) and 87 500 (form V). Form V predominated in extracts from severely affected fruit, along with form I and a minor form of approximate MW 117 500 (form IV). Interestingly, the elution profile of extracts from healthy fruit prepared in the presence of 0.1 % SDS was similar to that observed for mildly affected fruit; comparison with the profile for healthy fruit indicated: (a) the appearance of high MW form I; (b) the predominance of form II; and (c) the maintenance of low MW form VI, which was absent from mildly and severely affected fruit. It is possible that the causal events underlying the appearance of mesocarp discolouration result in the release of bound/latent forms of PPO which may initially exhibit similar chromatographic properties to those forms obtained from SDS-extracted healthy fruit (Fig. 1A); form VI may then be converted to form V (this possibly being a monomer to a dimer interconversion), the form found in affected fruit. Similarly, some of the intermediate MW forms may be convertible to the high MW form I. Such an interpretation is consistent with previous results on PPO interconversions [13, 17–19], but clearly detailed enzymological, protein chemical and immunological studies are necessary before firmer conclusions can be made. However, in the present work it was of interest that different multiple form patterns were observed in fruit with different symptoms, and forms I, II, IV and V were

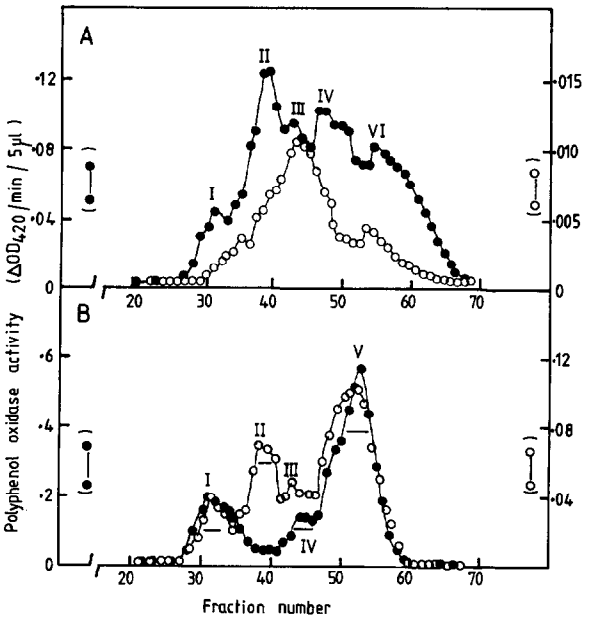

Fig. 1. Elution profiles for avocado PPO activity from healthy and mesocarp discolouration affected fruit following gel-filtration on a column of Sephacryl S-300. For each column run, 40 g of frozen pulp (5 g from each of eight separate fruit) was extracted in 80 ml of 10 mM sodium acetate, 7 mM 2-mercaptoethanol, pH 5.0. The 35–75% ammonium sulphate fraction was prepared, and the final pellet taken up in 5 ml of 10 mM sodium acetate. Then 1.5 ml of this extract (equivalent to 12 g fr. wt pulp) was applied to the column in each case. (A) Profile from healthy avocado pulp extract (○——○) plus profile for total PPO obtained by extraction of healthy pulp in the presence of 0.1% SDS (●——●). (B) Profiles from mildly affected (○——○) and severely affected (●——●) avocado pulp. Note that the profiles are drawn on different scales. Peaks I–VI were numbered in order of elution from the column. Fractions from peaks I, II, IV and V were pooled as indicated by the bars, and subjected to kinetic analysis.

subjected to kinetic analysis using a variety of substrates naturally occurring in avocado, in order to examine whether any relation existed between specific forms of PPO and endogenous phenolic pools present in fruit showing varying degrees of symptoms.

The results (Table 3) indicated that form II had the lowest K_m for chlorogenic acid, while forms I and IV exhibited no activity against this substrate. This is in contrast to the results reported for cv Fuerte by Kahn [16], where all six forms of PPO separated by PAGE appeared to oxidize chlorogenic acid. Forms I and V, the predominant forms in affected fruit, exhibited low K_m values for the oxidation of catechin, with form I showing the lowest K_m for epicatechin. None of the forms tested was capable of catalysing the oxidation of caffeic acid (cf. ref. [16]).

Qualitative analysis of polyphenols

Polyphenols in methanol extracts from healthy and affected fruit were separated by two-dimensional TLC according to the method of Ramirez-Martinez and Luh [20]. Several phenolic compounds were identified by: (a) a comparison of R_f values with those of ref. [20] and with standards run at the same time as the present analysis of fruit samples; (b) the appearance of spots under UV light of wavelength 254 and 366 nm; and (c) the appearance of spots after spraying plates with diazotized 4-nitroaniline reagent. Compounds identified included both the *trans*- and *cis*-isomers of caffeic acid, 4-coumaric acid and chlorogenic acid, and also catechin and epicatechin. Far greater amounts of caffeic and 4-coumaric acids were found in mildly and severely affected, as compared with healthy fruit, and epicatechin was completely absent from chromatograms of extracts from severely affected fruit.

Collectively, these results allow speculative conclusions to be drawn concerning symptom expression associated with mesocarp discolouration. Firstly, the browning reaction observed as a result of cutting the fruit, thus allowing exposure of internal tissues to oxygen, results from the presence in the tissues of high PPO activities and, possibly, higher levels of PO activity and phenolic substrates. The differences in PPO activity between affected and healthy fruit probably result from the release of bound PPO and/or activation of latent PPO rather than increased synthesis of the enzyme in response to the initial cause of the disorder. The forms of the enzyme found in affected fruit may be multimers of the forms found in healthy fruit. The substrate specificities of the PPO forms in affected fruit suggest that: (a) catechin and epicatechin may be the preferred substrates for the browning reaction; and (b) caffeic acid, although present in relatively high concen-

Table 3. Properties of PPO fractions obtained by Sephacryl S-300 gel-filtration of extracts from healthy and mesocarp discolouration affected avocado fruit

Fraction	Approx. MW	K_m (mM) 4-Methyl-catechol	Catechin	Epicatechin	Chlorogenic acid	V_{max} (ΔOD_{420}/min per 20 μl) 4-Methyl-catechol	Catechin	Epicatechin	Chlorogenic acid
I	500000	1.92	0.14	1.52	NA	0.29	0.13	0.36	NA
II	230000	1.22	0.74	3.50	1.05	1.11	0.47	0.21	0.37
IV	117500	1.00	ND	15.39	NA	0.09	ND	0.09	NA
V	87500	2.2	0.35	6.25	7.14	1.33	0.57	1.0	2.0

MWs were estimated by reference to a calibration curve for the Sephacryl column. The K_m and relative V_{max} values were calculated from double reciprocal plots of the initial rate data taken at six or more substrate concentrations. NA = no activity; ND = not determined.

trations, may not be oxidized by PPO after cutting affected fruit. The absence of epicatechin in extracts of severely affected fruit might have resulted from oxidation of this substrate prior to extraction of the tissues, or alternatively from rapid oxidation during the cutting and pulping of the fruit samples prior to freezing. The increased levels of hydroxycinnamic acids in affected fruit indicate stimulation of the general phenylpropanoid pathway following the initial event which leads to the disorder, although by the time the fruit was inspected the PAL activity levels may have declined to a level where no significant differences could be observed between healthy and affected fruit. Future work will be concerned with identifying the physiological conditions responsible for triggering the biochemical changes which lead to mesocarp discolouration, and also with the biochemical and physiological relationships between the mesocarp discolouration and pulpspot disorders.

EXPERIMENTAL

Source of experimental material. Avocado fruit (cv Fuerte), exported by boat from South Africa, were collected at Covent Garden Market, London, during April–June 1983. Fruit were ripened in the dark at 22°, cut in half longitudinally, and the cut surfaces exposed to the atmosphere at room temp. for 30 min prior to assessment of symptoms. Fruit were classified as: (a) healthy, if no browning of the pulp was observed; (b) mildly affected if localized areas of light grey-brown discolouration appeared; and (c) severely affected if grey-brown discolouration of the whole distal region of the pulp was observed. Discoloured surface material was removed with a knife, and the fruit were rapidly peeled and placed in plastic bags under an atmosphere of N_2. The bags were sealed and the fruit mashed to a uniform consistency before being frozen at −70° until required for analysis.

Enzyme extractions and assays. PPO and PO were extracted from 2 g batches of thawed pulp by homogenization in a pestle and mortar with 0.2 g PVP and 10 ml NaOAc buffer, pH 5.0 at 0°. PAL was extracted in a similar manner but using 5 ml 50 mM Tris–HCl, 1.4 mM 2-mercaptoethanol, pH 8.5. Extracts were squeezed through four layers of muslin, and centrifuged at 15000 *g* for 15 min at 4°. PPO was assayed by the addition of 10 μl supernatant to a mixture of 5 ml 10 mM NaOAc, pH 5.0 and 5 ml 0.02 M 4-methyl catechol, the initial rate being measured at 420 nm and 25°. PO was assayed by the addition of 0.5 ml supernatant to a mixture of 10 ml 10 mM NaOAc, pH 5.0, 0.5 ml 0.04 M guaiacol and 0.5 ml 0.1 M H_2O_2. The initial rate was again measured at 420 nm and 25°. PAL was assayed spectrophotometrically as described [21]. Units of specific activity for PPO and PO are given as ΔOD_{420}/min per mg protein, PAL activities being expressed as μkat/kg protein (1 kat = 1 mol/sec).

Analysis of total phenols and proanthocyanidins was performed as described previously [22]. Catechol and grape leucocyanidin were used as standards for total phenol and proanthocyanidin determinations, respectively.

Qualitative analysis of polyphenols was by 2-D TLC (silica gel G/UV_{254}; 1st dimension *n*-BuOH–HOAc–H_2O, 4:1:5 (top layer) 2nd dimension 2% aq. HOAc) according to the method of ref. [20]. Compounds were observed under UV light at wavelengths of 254 and 366 nm and then the plates sprayed with diazotized 4-nitroaniline reagent [23]. The colours of spots and R_f values were compared with those of a set of known standards and with the data in ref. [20].

Protein was determined by the method of ref. [24].

Chromatography on Sephacryl S-300 was effected on a 1.3 × 68 cm column equilibrated with 10 mM NaOAc, pH 5.0. 1.5 ml of partially purified avocado PPO extract was applied to the column, and the sample eluted with 10 mM NaOAc at a flow rate of 0.25 ml/min, 1.6 ml fractions being collected. The column was calibrated with ribonuclease A (MW 13680), ovalbumin (MW 45000), bovine serum albumin monomer (MW 66000) and dimer, haemoglobin (MW 68000) and phenylalanine ammonia-lyase (MW 330000).

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