Phytochemistry, 1976, Vol. 15, pp. 267–272. Pergamon Press. Printed in England.

POLYPHENOL OXIDASE ISOENZYMES IN AVOCADO*

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(*Revised received* 26 *August* 1975)

Key Word Index—*Persea americana*; Lauraceae; avocado; polyphenoloxidase; isoenzymes.

Abstract—Avocado polyphenol oxidase (PPO) was precipitated mainly in the 30–90% saturated ammonium sulfate fraction. The 40–75% saturated ammonium sulfate fraction (the partially purified enzyme) had the highest specific activity in the cultivars Lerman, Horeshim and Fuerte. The PPO was active towards *o*-dihydroxyphenols. Six active enzymes (*a–f*) were detected with D,L-DOPA, 4-methylcatechol, catechol, caffeic acid or chlorogenic acid. Band *e* was the most active in all cases. More isoenzyme bands (fast-moving) were observed with caffeic acid than with 4-methylcatechol. Furthermore, the isoenzyme patterns of the partially purified extracts of the cultivars could be distinguished with respect to caffeic acid.

INTRODUCTION

We reported previously that the amount of polyphenol oxidase† present in cvs Lerman, Horeshim and Fuerte avocado correlates with the degree of browning occurring in the freshly cut fruits [1]. The total and the specific activity of polyphenol oxidase extracted was, in decreasing order, from cvs Fuerte, Horeshim, Lerman. Moreover, it is unlikely that these differences are due to the presence of inhibitors or activators [1].

Thomas and Nair showed that PPO activity differed considerably among three banana varieties and that gamma irradiation, which induces skin browning in banana, was positively correlated with an increased activity of isolated PPO [2]. Likewise, a good correlation was shown between tissue browning and PPO activity in irradiated mango fruits [3]. The degree of browning of some varieties of peaches was correlated with the tannin content of the ripe fruit and, in some cases, with the total PPO activity. Differences in the affinity of the enzyme for the substrate were reported [4, 5].

Isoenzyme patterns can be used to differentiate between species [6] and the isoenzyme components may be important in metabolic regulation [6–9]. Dizik and Knapp [10] have recently shown that avocado PPO exists in multiple forms; its isoenzymes ranged in MW from 14000 to 400000 with that of 28000 being the major one. The present study was undertaken to elucidate further the nature of the avocado PPO complex.

RESULTS AND DISCUSSION

The activity of the crude Lerman enzyme obtained from the fresh ripe fruit or from the acetone powder prepared therefrom was too low to enable convenient study of its kinetic properties or determination of its isoenzyme pattern. It was therefore necessary to purify the enzyme further. PPO from Lerman and from Fuerte could be precipitated at between 30 and 90% ammonium sulfate. While the specific activity of the former increased by only 20%, that of the latter increased by almost threefold. Less than 10% of the PPO activity precipitated in the 0–30% fraction in either variety.

Polyacrylamide gel electrophoresis of the 30–90% ammonium sulfate fraction

Samples of the 30–90% ammonium sulfate fraction were analyzed by polyacrylamide gel electrophoresis. Similar amounts of proteins of each sample were applied to the gel and PPO activity was detected by incubating the gels with either 4-methylcatechol or caffeic acid. Under the conditions tested, several apparently similar PPO isoenzymes were revealed in both the Lerman and the Fuerte PPO with additional PPO active bands detected in Fuerte PPO. Similar PPO isoenzyme patterns were also obtained with samples of homogenate of the fresh ripe fruit or the crude supernatant extracted from the acetone powder, prepared as described previously [1], indicating that the bands in the purified fractions were not an artifact caused by acetone or $(NH_4)_2SO_4$ precipitation. This conclusion was further supported by analyzing the crude and the partially purified avocado PPO by gel electrofocusing (see below), where the same isoenzyme pattern was obtained with the crude and the partially purified fractions. These results differ from those of Benjamin and Montgomery obtained with cherry PPO, where acetone precipitation caused changes in the isoenzymic pattern [11].

The effect of substrate on PPO isoenzyme pattern in the 30–90% ammonium sulfate fraction

Like many other plant PPO [5, 8, 12], avocado PPO is mostly active toward *o*-diphenols. It has already been

* Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. 1975, Series, no. 114-E.

† Abbreviations used: PPO: polyphenol oxidase (*o*-diphenol: O_2 oxidoreductase, EC 1.10.3.1, also known as phenolase, phenol oxidase, catechol oxidase and tyrosinase); D,L-DOPA: 3,4-dihydroxyphenylalanine; dopamine: 3,4-dihydroxyphenylethylamine.

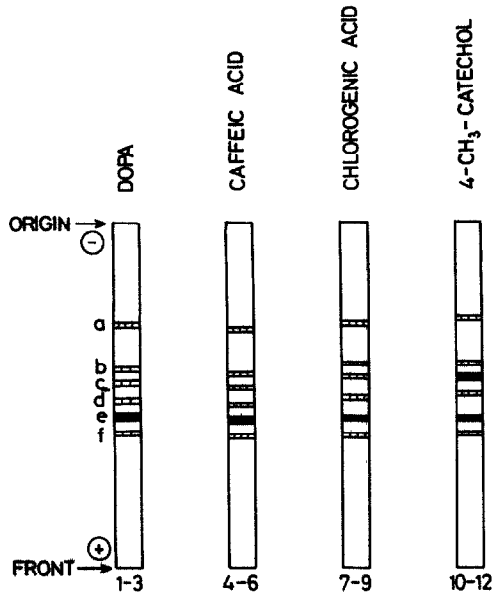

Fig. 1. Electrophoretic pattern of the 30–90% ammonium sulfate fraction of Fuerte avocado with different substrates. The dialyzed 30–90% $(NH_4)_2SO_4$ fraction of Fuerte avocado was applied to four groups (three gels each) as follows: 43, 86, 172 μg protein (corresponding to 0·85, 1·7 and 3·5 PPO activity units respectively) to gels 1–3, 4–6, 7–9 and 10–12. At the end of the run, the gels were stained with DOPA (1–3), caffeic acid (4–6), chlorogenic acid (7–9), or 4-methylcatechol (10–12), as described in Experimental. Identical isoenzyme patterns were obtained for the three levels of enzyme and only one of each group of three gels is shown.

shown [10, 13] that both crude and purified avocado PPO preparations have high affinity for catechin, dopamine and 4-methylcatechol, less so for catechol, pyrogallol and chlorogenic acid, and low affinity for D,L-DOPA.

The affinity of the PPO isoenzyme for different *o*-dihydroxyphenols was tested in an attempt to differentiate between the relative activity of the various isoenzymes of the Fuerte and the Lerman PPO enzyme. Equal amounts of proteins of the 30–90% $(NH_4)_2SO_4$ fraction were analyzed by polyacrylamide gel electrophoresis under identical conditions and the relative affinities of the isoenzymes toward 4-methylcatechol, chlorogenic acid, caffeic acid, and D,L-DOPA were tested. Six active isoenzymes were detected with each substrate (Fig. 1). In each case band *e* was the most active, while bands *a*, *b*, *c*, *d*, and *f* were similar to each other in their color intensity, except when 4-methylcatechol was the substrate—in which case band *c* was almost as active as band *e*. Using identical conditions, the time required for the appearance of PPO isoenzymes with each substrate was, in increasing order, as follows: D,L-DOPA, 4-methylcatechol, catechol, caffeic acid, and chlorogenic acid. Storing the gels in a solution of 10 nM ascorbic acid stabilized the caffeic acid and chlorogenic acid bands, but did not aid appreciably in either stabilizing or decreasing the high background color of gels stained with 4-methylcatechol or D,L-DOPA. Sheen [14] reported that in the presence of peroxides, peroxidase could oxidize chlorogenic acid and caffeic acid and warns of possible artifacts in detecting PPO on gels polymerized by ammonium persulfate, since residual peroxides can be derived from the latter after polymerization. In order to test this possibility, ammonium persulfate was substituted by riboflavin and Fuerte and Lerman samples were analyzed as otherwise described in Experimental. The same PPO isoenzyme pattern was obtained in the case of these two polymerizing agents, thus ruling out such possible artifacts. The same isoenzyme pattern was obtained by chromatographing the 30–90% $(NH_4)_2SO_4$ fraction of the Lerman and the Fuerte PPO enzymes. Thus, the Lerman and Fuerte PPO enzymes precipitated between 30 and 90% $(NH_4)_2SO_4$ could not be differentiated on the basis of the relative intensities of the isoenzyme bands to the substrates tested.

Further fractionation of avocado PPO by $(NH_4)_2SO_4$

Further $(NH_4)_2SO_4$ fractionation of Fuerte, Horeshim or Lerman revealed that 85–98% of the PPO activity of Fuerte and Horeshim could be recovered in the 40–75% fraction but only about 60% of the Lerman enzyme. In all cases, the sp. act. of PPO in the 40–75% fraction increased 2 to 3 fold compared with the sp. act. of the crude enzyme. Attempts to fractionate further the proteins of the highest PPO sp. act. (namely, those that precipitate between 40 and 75% $(NH_4)_2SO_4$ saturation) into 40–55% and 55–70% fractions revealed that PPO activity existed about equally in these narrower fractions. Here again, the sp. act. of the Fuerte PPO was about 10–20 fold higher than that of the Lerman. The PPO isoenzyme pattern of the 40–55% and the 55–70% fractions obtained from the Lerman avocado was very similar to that obtained from the Fuerte avocado. Whereas in the 30–90% $(NH_4)_2SO_4$ fraction isoenzymes of the same mobility were observed with either 4-methylcatechol or caffeic acid, in the 40–75% fraction (as well as in the 40–55% and 55–75% fractions) additional fast-moving isoenzymes were observed with caffeic acid but not with 4-methylcatechol. All further work was conducted on the dialyzed 40–75% $(NH_4)_2SO_4$ fraction only. This fraction will be referred to as the partially purified PPO enzyme.

DEAE-cellulose chromatography of Fuerte and Lerman PPO

Partially purified Lerman or Fuerte PPO was chromatographed on DEAE-cellulose. An aliquot from each fraction was assayed spectrophotometrically for PPO activity using 4-methylcatechol as the substrate. The elution pattern of Fuerte PPO activity is shown in Fig. 2. Two major peaks of activity appeared, with more activity in peak B than C. A small peak of activity, labelled A in Fig. 2, can also be discerned. An almost identical pattern was obtained when the elution was performed with a linear gradient of Na_2SO_4. When the buffer system was 50 mM Tris, pH 7·7, and the elution was with 0–0·5 M KBr in this buffer, a slightly different pattern was obtained: Peak A was not visible and peaks B and C were of equal activity. Gel electrophoresis analysis of the PPO isoenzymes eluted in the peak tubes of region B and C in the elution profile (see Fig. 2) revealed a similar isoenzyme pattern after long incubations with either caffeic acid or 4-methylcatechol. Under the conditions described above, similar chromatographic patterns were also obtained with the partially purified Lerman PPO.

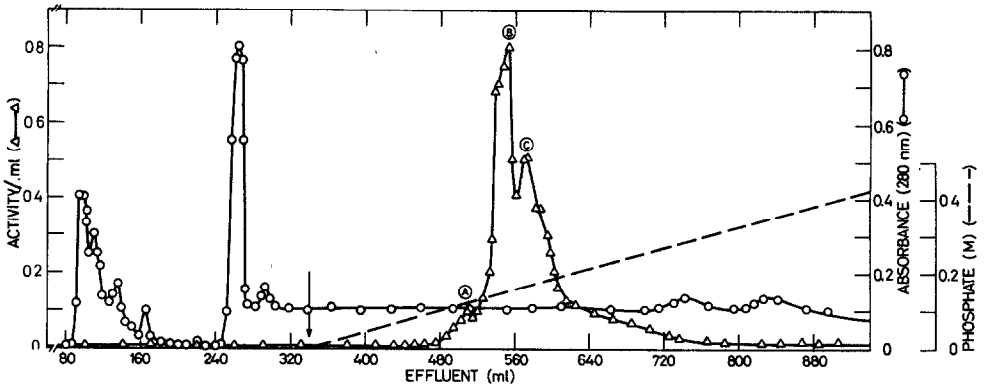

Fig. 2. DEAE-cellulose chromatography of the partially purified avocado PPO. The 40–75% $(NH_4)_2SO_4$ fraction of Fuerte avocado after dialysis (5 ml) containing 22·5 mg protein with 103 PPO units was applied to a 2·5 × 38 cm DEAE-cellulose column previously equilibrated with 50 mM sodium phosphate buffer, pH 6·5. The column was eluted with 330 ml of the same buffer and then (as indicated by the arrow) a linear gradient consisting of 300 ml 0·1 M NaPi buffer, pH 6·5, and 300 ml 0·4 M NaPi buffer, pH 6·5, was used for elution (the phosphate molarity is indicated by the dashed line). An aliquot of each fraction was assayed spectrophotometrically for PPO activity in the standard reaction mixture using 4-methylcatechol as the substrate.

PPO of Royal Ann cherries has also been resolved into two closely associated peaks following chromatography on DEAE-cellulose [11]. The first peak consisted predominantly of slow and intermediate isoenzyme bands and the second peak of fast-moving isoenzymes [11]. Similarly, catechol oxidase from apple chloroplasts was resolved by DEAE-cellulose chromatography into three peaks [15]. When gel electrophoresis analysis was conducted immediately after chromatography, a sample from each peak showed up as a single active band with different mobility for the band of each peak [15]. The majority of the PPO activity of Bartlett pears [12] was eluted from DEAE-cellulose column with 0·15 to 0·2 M Pi (fraction A) and a relatively small amount (fraction C) with 0·5 M Pi (pH 6·2). Electrophoresis on polyacrylamide gel showed two active PPO bands in fraction A and a single and different band in fraction C [12].

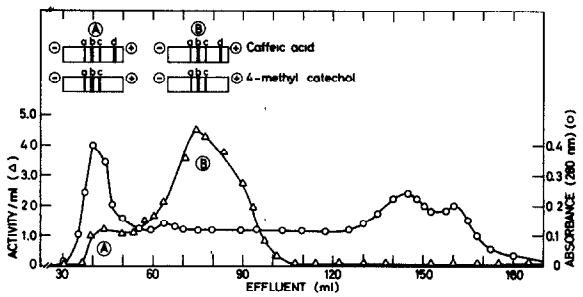

Fig. 3. Sephadex G-100 chromatography of the partially purified avocado PPO. The 40–55% $(NH_4)_2SO_4$ fraction obtained from Fuerte avocado (2 ml) containing 6·5 mg protein with 130 PPO units was applied to a 2·5 × 38 cm Sephadex G-100 column previously equilibrated with 0·05 M NaPi buffer, pH 6·5. The $A_{280\,nm}$ was measured (●——●) and an aliquot of each fraction was assayed for PPO activity using 4-methylcatechol as the substrate by the standard spectrophotometric assay (Δ——Δ). The peak tubes from fractions A and B were pooled separately and concentrated. The PPO isoenzyme pattern of each sample (A and B) was analyzed by gel electrophoresis as described in Experimental using either caffeic acid or 4-methylcatechol as the substrate. Bands *a*, *b* and *c* were seen much sooner than band *d*. The intensities of the bands was *b*, *d* > *a*, *c*. Under identical conditions, the same isoenzyme pattern was obtained with a sample of the 40–55% $(NH_4)_2SO_4$ fraction applied to the Sephadex G-100 column.

Chromatography of the partially purified Fuerte and Lerman PPO on Sephadex G-100

Chromatography on Sephadex G-100 was performed with either the 40–55% (I) or the 55–70% (II) dialyzed $(NH_4)_2SO_4$ fractions of the Fuerte avocado. The pattern of the PPO activity when either $(NH_4)_2SO_4$ fraction I or II was chromatographed by Sephadex G-100 column, revealed two activity peaks (Fig. 3). The elution pattern of $(NH_4)_2SO_4$ fraction I or II of the Fuerte PPO, and that of the 40–75% $(NH_4)_2SO_4$ fraction of the Lerman PPO, were very similar and only one pattern is therefore shown for illustration. Peak A (about 10% of the total activity) was eluted with the void volume of the column; peak B (about 90% of the PPO activity) was eluted later, with a peak around fraction number 40. Essentially all the PPO activity originally applied to the column was recovered in the effluent of both peaks A and B. The sp. act. of the pooled tubes in peak A was lower than that originally applied to the column (13 vs 20 A/410 nm/min/mg protein, respectively), while that of the pooled tubes in peak B was 4–5 fold higher than that originally applied. On the basis of preliminary determination of the elution volume for hemoglobin (MW 67000) and peroxidase (MW 40000) on the Sephadex G-100 column used, the MW of the bulk of the avocado PPO (peak B) was estimated to be roughly 35000.

The behavior of avocado PPO on Sephadex G-100 described above is different from that observed by Dizik and Knapp using the technique of thin layer gel filtration on Sephadex G-150 [10]. These investigators showed that a partially purified avocado PPO was resolved into five fractions of MW around 14000 (α), 28000 (β), 56000 (λ), 112000 (δ), and over 400000 (ϵ), with the β and ϵ being the major and minor fractions, respectively [10].

Peaks A and B obtained by column chromatography on Sephadex G-100 (Fig. 3) were concentrated separately with lyphogel and samples of each, as well as of the original material prior to chromatography, were analyzed by gel electrophoresis. The upper part of Fig. 3

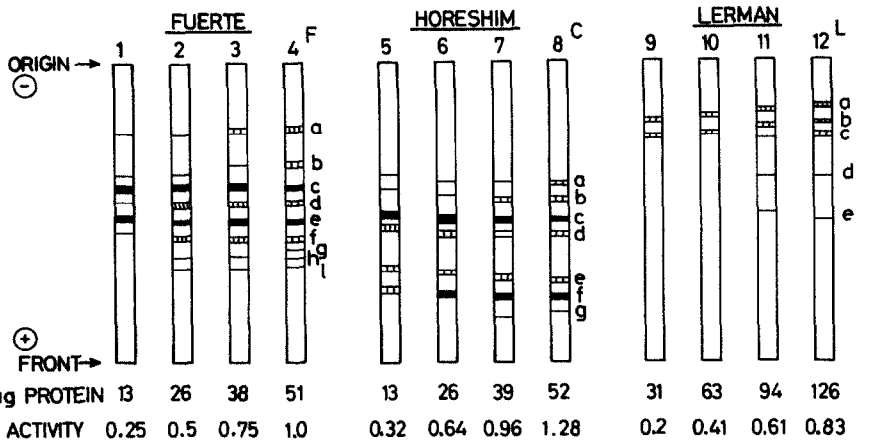

Fig. 4. Polyacrylamide gel electrophoresis of the partially purified PPO fraction of Fuerte, Horeshim and Lerman avocado. Samples of the 40–75% $(NH_4)_2SO_4$ fraction prepared from the 3 avocado varieties were applied in amounts and activities as indicated. Polyacrylamide gel electrophoresis and staining of the gels at the end of the run with caffeic acid were carried out as described in Experimental.

illustrates that under the conditions tested, the same isoenzyme pattern was obtained with samples in either peak. Three PPO isoenzymes *a*, *b*, and *c*, were revealed with 4-methylcatechol, with band *b* being the major one. Four bands appeared with caffeic acid, with band *d* appearing much later than *a*, *b* or *c*. After a relatively long incubation period (about 1 hr), bands *b* and *d* were of equal intensity.

Using the technique of thin layer gel filtration in one direction followed by electrophoresis in the second direction, Dizik and Knapp have detected six PPO isoenzymes of MW 28000, but concluded that only one of these contributed significantly to the color development measured in the spectrophotometric assay of total avocado PPO [10].

Like avocado PPO, cherry PPO can also be resolved on Sephadex G-100 into two peaks [11], with the exception that the ratio between the PPO of relatively high MW to that of low MW was much higher in cherry than in avocado. Moreoever, different isoenzyme patterns were observed in the two peaks of cherry PPO, as compared with identical patterns in the two avocado peaks (Fig. 3). Catechol oxidase from apple chloroplasts was resolved on Sephadex G-100 into three peaks—I, II & III—in the order of their elution. The MW of the bulk of its activity (peak II) was calculated to be between 60 and 70000 [15]. The PPO of each of these peaks showed up as a single band on gel electrophoresis, with fast-, medium-, and slow-moving bands in peaks I, II and III, respectively [15].

Electrophoresis and electrofocusing of partially purified Fuerte, Horeshim and Lerman PPO

When equal amounts of total *activity* of the partially purified enzyme from each variety were applied to polyacrylamide gels, differences in isoenzyme patterns became apparent (Fig. 4). The isoenzyme pattern for Fuerte enzyme, which had the highest sp. act. of the three varieties, revealed nine PPO active bands designated F–a through F–i, with F–c and F–e being the most intense ones. In the case of the Horeshim, which had intermediate PPO, sp. act. seven PPO active bands were obtained (H–a through H–g), with bands H–c and H–f being the major ones. Lerman PPO, which has the lowest sp. act. of the three avocado varieties studied, yielded only five active isoenzyme bands when comparable amounts of activity were put on the gels as for the other two varieties. These were designated L–a through L–e, with bands L–a, L–b and L–c being the major ones. Thus, it can be concluded from the data in Fig. 4 that each of the three avocado varieties has its own characteristic isoenzyme pattern. Lerman isoenzymes moved more slowly on the gels than those of Horeshim, while the Fuerte isoenzyme pattern seems to include several isoenzymes comparable to those seen in both Horeshim and Lerman.

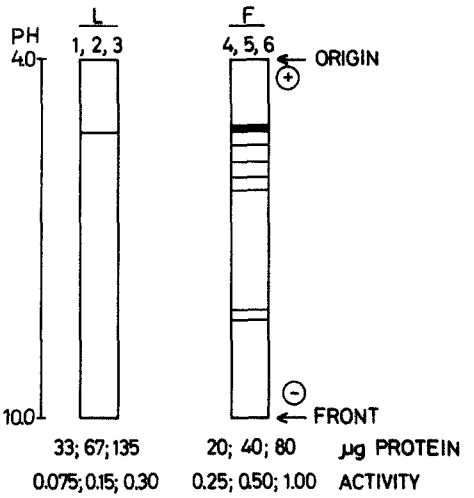

Fig. 5. Polyacrylamide gel electrofocusing of PPO from Lerman and Fuerte avocado. The 40–75% $(NH_4)_2SO_4$ fraction (i.e., the partially purified fraction) was applied to the gels. L and F refer to Lerman and Fuerte avocado, respectively. Three samples of each fraction, in increasing amounts of proteins, were applied to gels 1–3, 4–6, as indicated. Polyacrylamide gel electrofocusing with a pH gradient of ampholine between 4 and 10 in the direction as indicated, was carried out as described in Experimental. At the end of the run the gels were stained for PPO activity using 4-methylcatechol as the substrate. The same isoenzyme pattern was obtained within the set 1–3 and the set 4–6, and therefore only one pattern of each set is illustrated in the diagram.

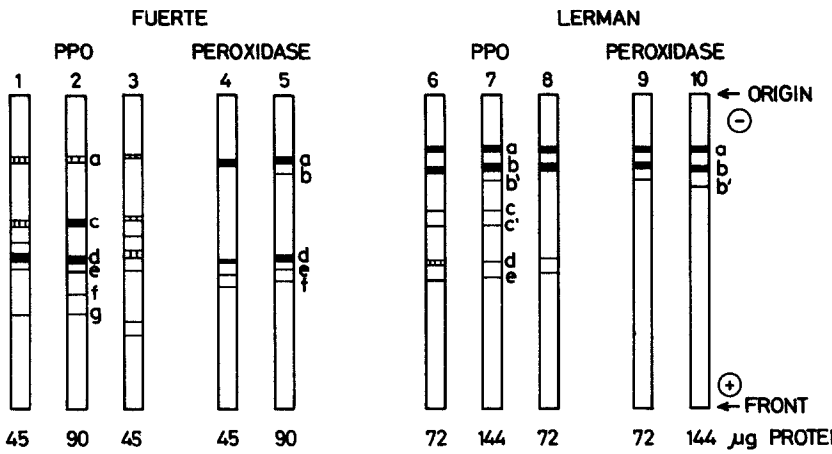

Fig. 6. PPO and peroxidase isoenzyme in the partially purified Fuerte and Lerman enzymes. Samples of the 40–75% $(NH_4)_2SO_4$ fraction of Fuerte and Lerman avocado were applied to the polyacrylamide gels 1–5 and 6–10, respectively, in the amounts indicated. PPO activity with 4-methylcatechol as the substrate was 0·43 (gels 1, 3, 4), 0·86 (gels 2, 5), 0·27 (gels 6, 8, 9) and 0·54 (gels 7, 10). Peroxidase activity with guaiacol and H_2O_2 as the substrates was 0·004 (gels 4, 9) and 0·008 (gels 5, 10) units. Total PPO and peroxidase activities were determined spectrophotometrically as described in Experimental. Electrophoresis was conducted as described in Experimental. At the end of the run, gels 1, 2, 6 and 7 were stained with caffeic acid for PPO isoenzymes. Gels 3 and 8 were incubated in the presence of caffeic acid together with H_2O_2. Gels 4, 5, 9 and 10 were stained for peroxidase isoenzymes. For futher details, see Experimental. Intensity of the bands: high ■; medium □□□□; low □.

The PPO isoenzyme in samples of the partially purified enzymes was also separated by gel electrofocusing between pH 4 and 10. Fig. 5 demonstrates that seven isoenzymes with 4-methylcatechol activity appeared in the Fuerte enzyme with a predominant slow isoenzyme, while under identical conditions only one isoenzyme, probably corresponding to the above predominant band, was revealed in the case of the Lerman enzyme. The partially purified Fuerte and Lerman PPO could further be differentiated on the basis of the isoenzyme patterns obtained by gel electrophoresis and stained with caffeic acid as substrate. Isoenzymes *c* and *d* predominate in Fuerte, while *a* and *b* predominate in Lerman (Fig. 6).

In another attempt to differentiate between the Lerman and the Fuerte PPO activity, studies were performed to determine whether the PPO isoenzymes were associated with peroxidase activity, as has been demonstrated in various biological systems [16]. Preliminary examinations showed that peroxidase activity was associated with the partially purified PPO enzyme of both Fuerte and Lerman avocado. Since the ratio of PPO activity (using 4-methylcatechol) of the Fuerte vs Lerman was about 4:1, and the ratio of peroxidase activity (using guaiacol, and H_2O_2) was only 2:1, it appears that PPO and peroxidase activity were not identical. To clarify the relation between PPO activity and peroxidase activity further, gel electrophoresis was conducted in duplicate and at the end of the run one gel was stained for PPO and its replicate for peroxidase activity as described in the legend to Fig. 6. Several peroxidase isoenzymes were detected in the Lerman or the Fuerte partially purified enzyme (Fig. 6). As might be expected from other systems [16], avocado PPO and peroxidase activity were associated in some of the bands. However, in the case of the Fuerte enzyme bands *c* and *d* predominated as PPO isoenzymes, while bands *a* and *d* predominated as peroxidase isoenzymes. In the case of the Lerman, on the other hand, the major peroxidase and PPO bands (*a* and *b*) overlapped.

Interconversion of PPO subunits, as reflected by changes in isoenzyme pattern on gel electrophoresis, is known to occur under a variety of conditions. Thus, for example, storage causes interconversion of potato PPO [17], chloroplast PPO [15], and mushroom tyrosinase [7]. The presence of detergents, changes in ionic strength, and protein concentration can also cause such interconversion. Therefore, care was taken throughout this study to compare the isoenzyme pattern of Fuerte and Lerman PPO under closely similar conditions.

EXPERIMENTAL

Crude polyphenol oxidase was extracted from the Me_2CO powder of cv. Fuerte, Horeshim or Lerman avocado varieties with 0·1 M NaPi buffer, pH 6·5 (standard buffer) as described in ref. [1]. $(NH_4)_2SO_4$ fractionation was carried out at 0°. Solid $(NH_4)_2SO_4$ was added slowly with stirring to the crude enzyme fraction without pH adjustment until the desired saturation was achieved. After stirring for 90 min, the suspension was centrifuged at 15000 *g* for 15 min and the pellet was separated from the supernatant. The pellet was resuspended in a small vol of 50 mM NaPi buffer, pH 6·5, dialyzed 12 hr against the same buffer, and then centrifuged. The supernatant was saved as the enzyme source and will be referred to as the partially purified PPO enzyme. The partially purified PPO of either Fuerte or Lerman avocado was stable for at least 3 days when kept at 4° in either NaPi buffer (50 or 100 mM pH 6 or 7 or in Tris HCl (50 or 100 mM), pH 7·5. The enzyme was routinely kept at 4° in 50 mM NaPi buffer, pH 6·5.

Assay of PPO activity. The spectrophotometric method described in ref. [1] was used. Unless otherwise specified, PPO activity was assayed in a standard reaction mixture that consisted of (freshly mixed) 5 ml of 50 mM NaPi buffer, pH 6·5 and 5 ml freshly prepared 20 mM 4-methyl catechol. One unit of PPO activity is defined as 1ΔA 410 nm/min and sp. act. is defined as units/mg protein. Where indicated, the formation of other phenol oxidation products was followed at appropriate wavelengths as outlined in ref. [11].

Peroxidase activity was assayed at room temp. in 50-ml conical flasks containing 16 ml of 50 mM NaPi buffer, pH 6·5; 2 ml of 0·5% guiacol in 50% EtOH, and 2 ml of 0·3% H_2O_2.

The reaction was initiated by the addition of the enzyme and the flask was thereafter swirled continuously. A_{460} nm was measured at 15 sec intervals. The reaction was linear for at least 4 min and the velocity was calculated from the linear portion of the curve. One unit of peroxidase activity is defined as 1 ΔA_{460} nm/min.

Protein was determined by the method of ref. [18].

Polyacrylamide gel electrophoresis was performed by the method of ref. [19] using tubes of 5 mm i.d. filled to about 8 cm with 7·5% polyacrylamide in 0·37 M Tris–HCl, pH 8·9, as the resolving gel and 1 cm of 2·5% polyacrylamide in 60 mM Tris–HCl, pH 6·8, as the stacking gel. The electrode buffer was 10 mM Tris–glycine buffer, pH 8·3. Samples contained 10% sucrose and between 10 and 200 μg protein as specified. Preliminary expts showed sucrose had no effect on the overall PPO activity. Electrophoresis was carried out with 2·5 mA per tube with the anode at the bottom. After electrophoresis (about 3 hr), the gels were stained for PPO activity at 20° by a modification of the method of ref. [20]; the gels were equilibrated with 0·1 M Na citrate, pH 4·5, for 30 min, and then immersed in a soln of the same pH containing 10 mM substrate and 0·1% *m*-phenylenediamine, as a coupling agent, for 10–30 min, depending on the substrate used. For stabilizing the color of the stained bands and decreasing the non-specific background color, the gels were stored in a soln of 10 mM ascorbic acid. No PPO bands were detected when a sample of any of the PPO preparations was boiled for 5 min before analysis. Where indicated, proteins were stained with Coomassie blue and destained with HOAc–MeOH in H_2O as described in ref. [21]. Peroxidase isoenzymes were stained on the gel by the method of ref. [22] using benzidine–guaiacol–H_2O_2 as the substrate. In some cases dual staining of isoenzymes for both peroxidase and PPO was done on the same gel, as described by Srivastava *et al.* [16].

Polyacrylamide gel electrofocusing was carried out as described in ref. [23] on 7·5% polyacrylamide containing 1% ampholine (LKB, Sweden) in the pH range 3·5–10. Potassium persulfate was used to polymerize the gels. The protein sample was applied in 10% sucrose and covered with a protective layer of sucrose and ampholine. The anode contained 0·4% H_2SO_4 and the cathode 0·4% ethanolamine. The sample ran to the cathode at a current of 1–5 mA/tube (about 5 hr). Following electrofocusing, the gels were stained for PPO activity as described above under "gel electrophoresis".

Sephadex G-100 (2·5 × 38 cm column) was equilibrated with 50 mM NaPi buffer, pH 6·5. 2 ml of the 40–55%, the 55–70% or the 40–75% $(NH_4)_2SO_4$ fractions of either the Fuerte or the Lerman avocado were mixed with 0·2 ml saturated sucrose soln and applied to the column. Elution was performed with 50 mM NaPi buffer, pH 6·5, at the rate of 0·5 ml/min. 2 ml fractions were collected and an aliquot of each fraction was assayed for PPO activity by the spectrophotometric assay using 4-methylcatechol as the substrate.

Chromatography on DEAE-cellulose, (microgranular, Whatman DE-52, preswollen) was effected on a 2·5 × 38 cm column equilibrated with 50 mM NaPi phosphate, pH 6·5. 5 ml of the partially purified avocado PPO was applied on the column and eluted with the same buffer to wash all proteins that were not adsorbed to the DEAE-cellulose. A 600 ml linear gradient of Pi (0·1–0·4 M) was used for elution (40 ml/hr, 3 ml fractions) of the enzyme.

Acknowledgments—The excellent technical assistance of Miss Rina Sade and Mr. Ben-Zion Granit is gratefully acknowledged. We thank Kibbutz Horeshim and Mr. Meir Lerman of Gan Hayyim. Israel, for their generous supply of the avocado fruits.

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