Polyphenol Oxidase Activity and Browning of Three Avocado Varieties

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A comparison between the \textit{in vitro} total and specific activity of polyphenol oxidase of three avocado varieties, Fuerte, Horeshim and Lerman, showed clear differences that were correlated with the \textit{in vivo} rate of browning of the corresponding freshly cut mature fruit; Fuerte had the highest values, followed by Horeshim and then Lerman. A good correlation existed between the PPO activity in the crude homogenate of the fresh fruit and the crude enzyme extracted from the acetone powder prepared from each variety. The possibility that the relatively low PPO activity in the crude enzyme of the Lerman avocado was due to the presence of an inhibitor and to factor(s) degrading the enzyme, or that the relatively high activity of the Fuerte enzyme was due to an activator, was tested in various ways and ruled out. It was therefore concluded that the differences in the rate of browning of the three avocado varieties studied were directly related to the PPO activities as expressed in the crude enzyme fraction.

1. Introduction

The browning reaction in fruits and vegetables resulting from mechanical injury during postharvest storage or processing, is a widespread phenomenon. It is usually commercially undesirable because of the unpleasant appearance and the concomitant development of off-flavour. Browning is caused mainly by the oxidation of polyphenols present in the tissue and their subsequent polymerisation to \( o \)-quinone by both non-enzymatic and enzymatic reactions. The enzyme initiating this sequence of reactions is known by the general name of polyphenol oxidase or phenolase\( b \) (PPO) \((o\)-diphenol: oxygen oxidoreductase, ECI.10.3.1). Several recent comprehensive reviews\( ^1-4 \) discuss the role of polyphenols and polyphenol oxidase in the browning reaction. In various foods the rate of browning can be directly related to (a) polyphenol oxidase level; (b) endogenous polyphenol content of the tissue; or (c) a specific combination of both factors. To reduce browning during processing, according to current thought, it is usually considered most desirable to select varieties of fruit that contain low levels of polyphenols, rather than varieties with low levels of polyphenol oxidase,\( ^1,5 \) and

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\( ^b \) Abbreviations used: polyphenol oxidase: PPO; \( DL-3,4 \), dihydroxyphenylalanine: DOPA; 3,4, dihydroxyphenylethylamine: Dopamine; diethyldithiocarbamate: DETC.
apparently relatively little experience has been gained in the choice of fruits with low polyphenol oxidase levels to minimise browning reaction.

One of the major obstacles encountered when processing frozen avocados commercially is the high rate of browning, which usually becomes particularly pronounced after thawing. During the course of studies to find adequate techniques to preserve avocado halves by freezing, we learned about marked differences in the rates of browning of freshly cut Lerman, Horeshim, and Fuerte varieties of avocado. Knapp and Dizik and Knapp have recently shown that polyphenol oxidase is present in avocado and have characterised some of its properties. In order to test whether there was any correlation between the observed differences in rate of browning and the polyphenol oxidase content of the three varieties mentioned above, we have used their methods to measure the polyphenol oxidase activity. It was found that the amount of polyphenol oxidase activity present in Fuerte, Horeshim, and Lerman avocados correlates with the degree of browning observed in freshly cut fruit.

2. Experimental

Avocado (Persea americana Mill.) fruits of three varieties, Fuerte, Horeshim and Lerman, were hand-picked from marked trees grown locally. The percentage of oil in the avocado was determined by the method of Gazit and Spodheim, and oil content of the fruit was used as an approximate index of maturity. Only fruit considered to be commercially mature was used. After harvest, the fruits were kept at 24 °C until ripe (here we refer to ripe in terms of texture and eating quality). Acetone powder was prepared from ripe avocado by the method of Dizik and Knapp, with yields ranging between 10-15% of fresh flesh weight. PPO was extracted from the powder by suspending it in 0.1 M sodium phosphate buffer, pH 6.8 (5 mg powder/ml), and stirring continuously at 24 °C. Following extraction, the suspension was centrifuged at 15 000 x g for 20 min and the supernatant (referred to as the crude enzyme fraction) was used as the enzyme source.

2.1. Assay of polyphenol oxidase activity

The standard reaction mixture consisted of 5 ml of freshly mixed 0.1 M sodium phosphate buffer, at pH 6.5, and 5 ml of freshly prepared 0.02 M 4-methyl catechol; the absorbance at 410 nm was measured in a Gilford Model N-300 spectrophotometer. Reaction velocity was computed from the initial linear slopes of the curves obtained by plotting the optical density against time. PPO activity is expressed as absorbance at 410 nm/min, and specific activity as activity/mg protein. Where phenols other than 4-methyl catechol were used as the substrate, the change in absorbance at the appropriate wavelength was measured. Protein was determined by the method of Lowry et al.

3. Results and discussion

When mature, ripe avocados of the Fuerte, Horeshim and Lerman varieties were cut in half longitudinally and exposed to air, pronounced differences in their rate of browning were observed. External discoloration was visible within 1, 4-6, and 12-18 h in Fuerte, Horeshim, and Lerman, respectively. In the three varieties, darkening was
initially noticeable under the skin, within the seed cavity and around the conductive vessels. Eventually, the whole cut surface turned dark grey.

Using the crude enzyme fraction of each avocado variety, the velocity of the reaction was linear with time for at least 1 min with, for example, 4-methyl catechol, chlorogenic acid, caffeic acid, and catechol, and for at least 4 min with DOPA, Dopamine, and pyrogallol (each at a final concentration of 0.01 M).

PPO activity was measured at various enzyme concentrations and activity was computed from the linear portion of the curve. The crude PPO enzyme from either Fuerte, Horeshim or Lerman had a pH optimum between 5.3 and 6.7 with either 4-methyl catechol or DOPA as the substrate, and all measurements were therefore conducted at this pH range. The enzyme was stable for several days when kept at 4 °C in sodium phosphate buffer (0.1 or 0.05 M) at pH 6.0–7.0. The copper-binding agent DETC, a known inhibitor of PPO, strongly inhibited PPO activity of each of the three avocado varieties.

Preliminary experiments have shown that higher PPO activity was extracted at 24 than at 4 °C from the acetone powder of Fuerte, Horeshim or Lerman. Under the same conditions, total PPO activity of Fuerte avocado was high and that from Horeshim medium, compared with that from Lerman. Determination of the respective specific activity showed the same pattern as that of total extracted activity (Table 1). Over 50% of the total activity was soluble at the end of a 1 h extraction.

<table>
<thead>
<tr>
<th>Duration of extraction</th>
<th>Activity</th>
<th>Avocado variety</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lerman</td>
</tr>
<tr>
<td>1 h</td>
<td>Total</td>
<td>67.0</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>1.8</td>
</tr>
<tr>
<td>24 h</td>
<td>Total</td>
<td>102.0</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>3.3</td>
</tr>
</tbody>
</table>

The crude enzyme fraction was prepared from 400 mg acetone powder of each avocado variety and PPO activity assayed as described in the Experimental section.

The data indicated that a direct relationship seems to exist between the susceptibility of the avocado fruit to browning and the PPO activity in the crude enzyme.

The possibility that an artifact was formed in the course of preparing the acetone powder from either variety was ruled out on the following basis: a homogenate of fresh, ripe Lerman or Fuerte avocado was prepared and as can be seen in Table 2, the fresh homogenate of Lerman exhibits a much lower total activity as well as lower...
Table 2. PPO activity in fresh homogenates of ripe Fuerte and Lerman avocado

<table>
<thead>
<tr>
<th>Variety</th>
<th>Activity/ml</th>
<th>µg Protein/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuerte</td>
<td>11.0</td>
<td>480</td>
<td>22.0</td>
</tr>
<tr>
<td>Lerman</td>
<td>0.25</td>
<td>350</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Fresh, ripe avocado halves (cut longitudinally) were homogenised at 4 °C for 5 min with 0.1 M sodium phosphate buffer, pH 6.5 (1 g avocado/15 ml buffer), in a glass homogeniser. The homogenate was passed through cheese cloth, centrifuged at 15 000 × g for 15 min, and the supernatant obtained was used as the enzyme source.

The specific activity compared with that of Fuerte. Moreover, mixing aliquots of Fuerte and Lerman enzyme in varying ratios gave activities corresponding approximately to the sum of each assayed separately. Since similar results were obtained with either the homogenate of the fresh fruit or the supernatant of the acetone powder, all further experiments were conducted with acetone powder as the enzyme source.

The possibilities that the low PPO activity of the Lerman enzyme was due to the presence of an inhibitor, an inactivator or to factor(s) degrading the enzyme (e.g. endogenous proteolytic activity), while the high activity of the crude Fuerte enzyme was due to an activator, were investigated in various ways:

1. The effect of thorough dialysis of the crude enzyme from the two sources was tested and it was found that dialysis had no effect on the relative activities of the two enzymes (Table 3).

Table 3. Effect of dialysis on the PPO activity of Fuerte and Lerman avocado

<table>
<thead>
<tr>
<th>Variety</th>
<th>Treatment</th>
<th>Activity/ml</th>
<th>µg Protein/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuerte</td>
<td>Control</td>
<td>3</td>
<td>540</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>4.1</td>
<td>480</td>
<td>8.5</td>
</tr>
<tr>
<td>Lerman</td>
<td>Control</td>
<td>0.4</td>
<td>480</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Dialysis</td>
<td>0.82</td>
<td>360</td>
<td>2.2</td>
</tr>
</tbody>
</table>

A portion of the crude PPO fraction of each sample was dialysed overnight at 4 °C against 0.05 M sodium phosphate buffer, pH 6.5, centrifuged, and the PPO activity in the supernatant measured. The non-dialysed enzyme was kept overnight at 4 °C and assayed at the same time as its dialysed counterpart.

2. A mixture of crude enzyme from Lerman and Fuerte was prepared and aliquots of this mixture were immediately assayed.

The observed activity was very close to that expected from computation of the activity obtained when the corresponding amounts of each enzyme were assayed separately.
3. The activity of the Lerman enzyme was assayed for 30 s and then an aliquot of the Fuerte enzyme was added; the resultant activity was further determined. Likewise, mixing was done where aliquots of the enzymes were added in the reverse order. It was found that the activity where the enzymes of the two sources were mixed, was identical to that computed from assays where the activity of each enzyme was determined separately. There was no effect of the sequence of mixing the two enzymes nor of the ratio between them used in the specific assays.

4. Pre-incubation (at 0 or 24 °C) of the Fuerte enzyme with various amounts of the Lerman enzyme for various periods of time (tested for at least 20 min) prior to the initiation of the reaction by the addition of the substrate, had no effect on the expected activity.

5. The PPO activity extracted from a 1 : 1 mixture of acetone powder from Lerman and Fuerte was shown to be exactly as expected compared with that obtained when the activities were extracted from each source of acetone powder separately, this was true not only on the basis of determination of activity/ml enzyme, but also on the basis of determination of specific activities.

6. Addition of boiled Lerman enzyme had no effect on the activity of freshly prepared Fuerte PPO. Since boiling was likely to liberate an inhibitor from an enzyme-inhibitor complex present in the Lerman extract, this result appears to rule out the likelihood that the Lerman extract exhibited low activity because of the presence of an enzyme-inhibitor.

7. Addition of purified polyclar AT14 to the acetone powder, in a 1 : 1 ratio (w/w), had little effect on the extracted PPO activity of either Lerman or Fuerte, whereas the addition of a large excess of polyclar (5 : 1, w/w) resulted in a 35-50% increase in specific activity of the Lerman and Fuerte over that of the control (Table 4).

Table 4. Effect of polyclar on the recovery of PPO activity from acetone powder of Fuerte and Lerman avocado

<table>
<thead>
<tr>
<th>Variety</th>
<th>Addition during extraction</th>
<th>Activity/ml</th>
<th>μg Protein/ml</th>
<th>Specific activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lerman</td>
<td>None</td>
<td>1.2</td>
<td>206</td>
<td>5.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Polyclar, 1 : 1 (w/w)</td>
<td>1.3</td>
<td>210</td>
<td>6.2</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>Polyclar, 5 : 1 (w/w)</td>
<td>1.7</td>
<td>214</td>
<td>7.9</td>
<td>136</td>
</tr>
<tr>
<td>Fuerte</td>
<td>None</td>
<td>4.8</td>
<td>162</td>
<td>30.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Polyclar, 1 : 1 (w/w)</td>
<td>5.0</td>
<td>170</td>
<td>29.4</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Polyclar, 5 : 1 (w/w)</td>
<td>9.0</td>
<td>200</td>
<td>45.0</td>
<td>150</td>
</tr>
</tbody>
</table>

300 mg acetone powder prepared from avocado of each variety was extracted with 50 ml 0.1 M sodium phosphate buffer, pH 6.5, for 24 h at room temperature, in the absence or presence of 300 mg or 1500 mg polyclar (none, 1 : 1 and 5 : 1, respectively); centrifuged; and the supernatant dialysed overnight at 4 °C against 0.05 M sodium phosphate buffer, pH 6.5; centrifuged; and the activity of the supernatant assayed.

These observations seem to rule out the possibility that the Lerman enzyme contains an inhibitor or a factor that degrades the PPO enzyme, or that the Fuerte enzyme contains a PPO-activator factor. Further work is being carried out to characterise...
the PPO enzyme from these three avocado varieties in more detail. Moreover, the endogenous level and the distribution of polyphenols in the fruits are currently being analysed in order to test whether the PPO activity is the sole factor contributing to the differences observed in the rate of browning of the three avocado varieties.

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References