Purification and Some Properties of Two Polyphenol Oxidases from Bartlett Pears

NILO DE JESUS RIVAS AND JOHN R. WHITAKER
Department of Food Science and Technology, University of California, Davis, California 95616

ABSTRACT

Two polyphenol oxidases (enzymes A and B) from Bartlett pear (Pyrus communis) peelings were purified to electrophoretic homogeneity according to polyacrylamide gel by a combination of Sephadex gel filtration, diethylaminoethyl cellulose chromatography and hydroxylapatite chromatography. While the two enzymes differ electrophoretically at pH 9.3, chromatographically on hydroxyl apatite, and in the effect of ionic strength on activity, they are similar with respect to chromatography on diethylaminoethyl cellulose, substrate specificity, pH activity relations, inhibition by p-coumaric and benzoic acids, and heat stability. The two enzymes are o-diphenol oxidases with no detectable monophenolase or laccase activities. Pyrocatechol, 4-methyl catechol, chlorogenic acid, and d-catechin are good substrates of the enzymes with $K_m$ values in the range of 2 to 20 mM. Dependences of activity on oxygen and chlorogenic acid concentrations indicate a sequential mechanism for binding of these substrates to enzyme B. $V_{max}$ and $K_m$ values for oxygen and chlorogenic acid were 105 μmoles O$_2$ uptake per minute per milligram of enzyme, 0.11 mM and 7.2 mM, respectively, for enzyme B at pH 4.0. Both enzymes had maximum activity at pH 4.0 on chlorogenic acid. $K_m$ values for chlorogenic acid were independent of pH from 3 to 7; the $V_{max}$ values for both enzymes gave bell-shaped curves as a function of pH. p-Coumaric acid is a simple, linear noncompetitive inhibitor with respect to chlorogenic acid at pH 6.2 with $K_I$ values of 0.38 and 0.50 mM for enzymes A and B, respectively. Benzoic acid is a linear competitive inhibitor with respect to chlorogenic acid at pH 4.0 with $K_I$ values of 0.04 and 0.11 mM for enzymes A and B, respectively.

Polyphenol oxidase is also known as phenol oxidase, tyrosinase, o-diphenol oxidase, catechol oxidase, phenolase, and chlorogenic acid oxidase.

Brown discoloration of canned pears and pear concentrate was found to be related to the enzymatic browning that takes place before or during processing (18). Since polyphenol oxidase in Bartlett pear has not been adequately investigated, the purpose of this investigation was to purify the polyphenol oxidase complex of Bartlett pear and to determine its kinetic properties. An understanding of the factors which affect activity of the enzyme may lead to a better understanding of its function in vivo and to its control in the ripe fruit.

MATERIALS AND METHODS

Pears. Bartlett pears (Pyrus communis) at harvest maturity (19 p.s.i.) were obtained from an orchard in Placer County, California and ripened at 20 C and 85% relative humidity to a pressure test of 3 p.s.i. The ripe fruits were peeled, halved, and cored by hand. Peelings and flesh were quick frozen separately with Freon 12 in a duPont minimimark freezer, sealed in No. 2½ cans under a vacuum of 15 inches Hg and stored at −26 C until used. Under these conditions, the samples showed no darkening after 2 years storage.

Other Reagents. Sephadex G-25, particle size 20 to 80 microns, was from Pharmacia Fine Chemicals, Inc. DEAE-cellulose, medium mesh and ion-exchange capacity of 1.0 meq/gm, was from Sigma Chemical Co. Hydroxyl apatite (BioGel P-15) was from BioRad Laboratories. Polyethylene glycol (average molecular weight of 3000–3700) was from J. T. Baker Chemical Co. Acrylamide, bis-acrylamide, pyrocatechol, and resorcinol were from Eastman Organic Chemicals. Chlorogenic, caffeic, and p-coumaric acids were from Sigma Chemical Co. d-Catechin and phloroglucinol were from K and K Laboratories. Ferulic acid was from Nutritional Biochemical Corporation and 4-methyl catechol was from Aldrich Chemical Co. 4-Methyl catechol was recrystallized from toluene and d-catechin was recrystallized from water. All other substrates of reagent grade were used without further purification.

Purification of Enzyme. All steps were carried out at about 1 C. Two hundred and fifty grams of frozen pear peelings were homogenized in a Waring Blender with 500 ml of ice-cold 0.1 M sodium phosphate buffer, pH 6.2, containing 0.03 M ascorbic acid and 1% polyethylene glycol to slow down browning. The homogenate was centrifuged for 30 min at 10,500g in a Sorvall refrigerated centrifuge, and the supernatant liquid was filtered through glass wool.

The supernatant liquid in 100-ml aliquots was immediately placed on a column (4.1 × 37 cm) of Sephadex G-25 equilibrated with ice-cold 0.1 M sodium phosphate buffer, pH 6.2, containing 0.03 M ascorbic acid and 1% polyethylene glycol.

1 Taken from the dissertation submitted by Nilo de Jesus Rivas to the Graduate Division, University of California, Davis, in partial satisfaction of the requirements for the Ph.D. degree. N. J. R. gratefully acknowledges a scholarship from the University of Venezuela and a grant-in-aid for chemicals from the Graduate Division, University of California, Davis.

2 Present address: Facultad de Agronomía, University of Venezuela, Maracay, Venezuela.

3 Abbreviation: DEAE: diethylaminoethyl.
Fig. 1. Analytical DEAE-cellulose chromatography of pear polyphenol oxidases. The column (1.0 x 45 cm) and extract (following gel filtration) were equilibrated at 1 C with 1 mM phosphate, pH 6.2. Elution was with a linear gradient of 200 ml of 1 mM phosphate, pH 6.2, in mixing chamber and 200 ml of 0.25 M phosphate, pH 6.2, in reservoir. Proteins concentration (solid line) was determined by the Lowry method. Enzyme activity was determined in a reaction mixture containing 2.8 ml of 10 mM pyrocatechol in 10 mM sodium phosphate, pH 6.2, and 0.2 ml of enzyme solution. Activity is expressed as change in absorbance at 420 nm after 10 min reaction at 35.0 C.

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The gel tubes, 8.9 cm long and 0.5 cm i.d., contained 5.0 cm of running gel (7%) and 1.0 cm of spacer gel (1.25%). The enzyme solution was mixed with 70% sucrose and layered on top of the spacer gel. The starting pH was 8.3, and the running pH was 9.5. A current of 2 ma/tube was employed at 1 C until bromophenol blue, used as a reference marker, migrated close to end of the tube (about 4 hr).

Part of the gels were stained for protein with either 1% Amido black 10B in 7% acetic acid or with 0.01% Coomassie brilliant blue in 35% ethanol, 10% acetic acid, 55% water. Other gels, run at the same time as those for protein staining, were developed with one of the following substrate solutions: (a) 30 mM pyrocatechol in 0.2 M phosphate buffer-30% ethanol, pH 6.2; (b) 20 mM dicatechin in 0.2 M phosphate-30% ethanol, pH 6.2; (c) 5 mM pyrocatechol-5 mM L-proline in 0.1 M phosphate, pH 6.2.

**Protein Estimation.** The concentration of protein was determined by the method of Lowry et al. (11) using bovine serum albumin as standard. The micro-Kjeldahl method of Johnson (9) was used for crude extracts containing phenolic compounds.

**Enzyme Activity Measurements.** Polyphenol oxidase activity during purification of enzyme was determined by a modification of the colorimetric method described by Ponting and Joslyn (14). The substrate was 10 mM pyrocatechol in 10 mM sodium phosphate, pH 6.2. The increase in absorbance at 420 nm after 10 min incubation at 35 C was determined in a Beckman DB spectrophotometer.

Initially, the colorimetric method was also used in the kinetic studies. The change in absorbance at 420 nm versus time was recorded in a thermostated Beckman DB recording spectrophotometer. Initial velocities were calculated from the slope of the initial portion of the activity curves. Eventually, the colorimetric method was abandoned in favor of the polarographic method because (a) better initial rate curves were obtained, (b) less enzyme per reaction was needed, and (c) the change in oxygen concentration was more comparable among different substrates than was the colorimetric assay where the extinction coefficient of each quinone is different. Of the kinetic results reported here, only the Km values in Table II were determined by the colorimetric method.

Oxygen uptake during the enzymatic reaction was followed with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) coupled to a Model 80A Moseley autograp recorder and Model 53 Biological oxygen monitor. The electrode was standardized with air-saturated water at 30 C by adjusting the recording on the recorder chart to 100.

### Table I. Purification of Polyphenol Oxidases from Bartlett Pear Peelings

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific Activity</th>
<th>Activity Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units/ml</td>
<td>mg/ml</td>
<td>units/mg protein</td>
<td>% fold</td>
<td></td>
</tr>
<tr>
<td>1. Gel filtration</td>
<td>800</td>
<td>0.118</td>
<td>94.4</td>
<td>0.217</td>
<td>0.400</td>
<td>100</td>
</tr>
<tr>
<td>2. DEAE-cellulose</td>
<td>198</td>
<td>0.78</td>
<td>154</td>
<td>0.119</td>
<td>6.55</td>
<td>164</td>
</tr>
<tr>
<td>column, A’ (see Fig. 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Hydroxyapatite</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>column</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme A</td>
<td>275</td>
<td>0.14</td>
<td>38.5</td>
<td>0.0172</td>
<td>8.15</td>
<td>40.8</td>
</tr>
<tr>
<td>Enzyme B</td>
<td>152</td>
<td>0.22</td>
<td>33.4</td>
<td>0.0150</td>
<td>14.7</td>
<td>35.4</td>
</tr>
</tbody>
</table>

1 Change in absorbance at 420 nm/min·ml enzyme = 1 unit activity.
2 Based on activity following gel filtration. Activity measurements could not be performed on the supernatant liquid from the homogenate because of presence of ascorbic acid.

### Table II. Substrate Specificity of Bartlett Pear Polyphenol Oxidases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme A</th>
<th>Enzyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax</td>
</tr>
<tr>
<td></td>
<td>mM</td>
<td>pmol/min·mg protein</td>
</tr>
<tr>
<td>Pyrocatechol</td>
<td>20.9</td>
<td>17.2</td>
</tr>
<tr>
<td>4-Methyl catechol</td>
<td>8.0</td>
<td>22.7</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>16.1</td>
<td>18.4</td>
</tr>
<tr>
<td>d-Catechin</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Dopamine</td>
<td>3.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1.3</td>
<td>3.9</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

1 Fraction C (Fig. 2) had Km values of 22.5 and 1.5 mM on pyrocatechol and d-catechin, respectively.

At standard barometric pressure, this reading corresponds to an oxygen concentration of 0.24 mM. The reaction vessel, containing 3 ml of buffered substrate solution, was equilibrated in a water bath at 30 C until a steady rate was obtained. Then 0.05 ml of enzyme solution was introduced into the reaction vessel through a groove in the electrode by means of a calibrated syringe (No. 725 SN Hamilton syringe with a 4-inch needle). The reaction was allowed to proceed for 5 min. Initial velocities were determined from the initial linear part of the curves.

Vmax and Km values were determined from Lineweaver-Burk plots treated by the method of least squares using a Perkin-Elmer desk computer. The lines in Figures 5, 6, and 8 are drawn based on the least square treatment.

Only observed velocities at a single substrate concentration (v0) were determined for some substrates (Table II). The oxygen electrode method was used. The substrates were 10 mM in 0.2 M phosphate, pH 6.2. All solutions, except those of caffeic acid and dopamine, also contained 50 μM pyrocatechol in order to eliminate any lag period characteristic of the hydroxylation of monophenols (19).

Most of the kinetic studies were done in 25 mM succinate-25 mM pyrophosphate buffers with the ionic strength adjusted to 0.22 with NaNO3. These ions are not inhibitory of polyphenol oxidase activity (10).

**Effect of Enzyme Concentration.** The effect of concentration of enzymes A and B on measured activity was determined by both the colorimetric and oxygen electrode methods. Under the conditions used in this work and over the enzyme concentration range used, there was a linear relationship between enzyme concentration and activity.

**Oxygen Concentration Effect.** Gas mixtures of oxygen and nitrogen were prepared by means of two Nupro double pattern metering valves (Nupro Co., Cleveland, Ohio) placed at the out-
let of oxygen and nitrogen cylinders. Reaction mixtures were
equilibrated with different oxygen levels by bubbling the appro-
priate gas mixture through the reaction solution in the reaction
vessel for 10 min before adding the enzyme. Initial oxygen
concentrations were determined with the oxygen electrode.

**pH Stability of Enzyme B.** Aliquots of 0.1 ml of enzyme
solution, previously dialyzed against water, in 10 x 75 mm test
tubes were mixed with 0.4 ml of buffer solution ranging in pH
from 3.0 to 7.0. After incubation for 2 hr at 30 C, the remain-
ing activity was determined polarographically by adding 0.2
ml of incubated enzyme solution of 2.8 ml of 5 mm chlorogenic
acid at pH 4.0. For incubation and activity determinations, the
buffers were 25 mm succinate-25 mm pyrophosphate, \( \mu = 0.22 \).

**RESULTS AND DISCUSSION**

**Enzyme Purification.** Two proteins with polyphenol oxidase
activity were purified to homogeneity as determined by poly-
acrylamide disc gel electrophoresis. Homogeneity was achieved
in just three steps: gel filtration, DEAE-cellulose chromatog-
raphy, and hydroxylapatite chromatography (Table I).

Inclusion of 0.03 M ascorbic acid and 1% polyethylene gly-
col in the extracting fluid slowed down but did not completely
prevent browning following homogenization of the pear peel-
ings. Passage of the supernatant liquid through a Sephadex
G-25 column immediately after homogenization and centri-
fugation separated the enzyme from phenolic compounds and
gave a preparation that did not brown even after several days
in the cold.

Pear peelings were used in this work because they contain
substantially more polyphenol oxidase activity than the flesh.
The two enzymes combined represented only 7.2 x 10^-6% of
the peel weight (fresh weight basis), although they accounted
for approximately 10% of the extracted protein.

The recovery of activity following DEAE-cellulose chroma-
tography was more than 100% even excluding the activity
present in fraction C (Table I). This is most likely due to re-
moval of inhibitory material.

About 10% of the polyphenol oxidase activity was found
in a fraction, designated as C, eluted from DEAE-cellulose with
0.5 M phosphate (Fig. 2). On electrophoresis, the activity band
moved in an identical fashion to that of enzyme B. The \( \kappa_m \)
values of this fraction on pyrocatechol and \( d \)-catechin were
similar to those for enzyme B. On standing in the cold fol-
lowing elution from the DEAE-cellulose column, fraction C
gave a copious, gel-like white precipitate identified as pectic
material. Therefore, we conclude that fraction C is protein
from fraction A which had different chromatographic prop-
erties on DEAE-cellulose (Fig. 2) because it was complexed with
pectic material.

**Substrate Specificity.** The results of substrate specificity
studies of pear polyphenol oxidase enzymes A and B are shown
in Table II. The two enzymes are \( o \)-diphenol oxidases. No
cresolase or laccase activity was present as indicated by lack of
activity toward monophenols (\( p \)-coumaric and ferulic acids)
and \( m \)-phenols (resorcinol and chloroglucoinol). There was no
activity toward monophenols even in the presence of 50 \( \mu \)
pyrocatechol (19) or of 1 M ascorbic acid added to eliminate
the lag phase characteristic of hydroxylation of monophenols
by polyphenol oxidases. Some plant polyphenol oxidases, \( i.e. \)
mushroom, potato, broad bean, catalyze both the hydroxyla-
tion of monophenols and the oxidation of \( o \)-diphenols. How-
ever, many polyphenol oxidases lack monophenol oxidase ac-
divity (2, 13, 24).

Chlorogenic acid, \( d \)-catechin, pyrocatechol, and 4-methyl
catechol were all rapidly oxidized, although the last two com-
pounds do not normally occur in pears. The results lend sup-
port to earlier suggestions that chlorogenic acid and catechins
are the major substrates for pear polyphenol oxidase (16, 20).
Catechins, leucoanthocyanins, and chlorogenic acid consti-
tute about 90% of the total polyphenolic content of Bartlett
pears (17).

The \( \kappa_m \) values on all substrates were three to five times
higher for enzyme B than for enzyme A. Whether this means
enzyme B is a more efficient catalyst than enzyme A or whether
the active site concentration of B is greater than for A cannot
be determined from the data available. The \( \kappa_m \) values are
similar to those obtained with other fruit polyphenol oxidases
(6, 13, 24).

**Effect of Ionic Strength.** The activity of enzymes A and B
on pyrocatechol as a function of ionic strength, as adjusted
with NaNO\(_3\), was determined (Table III). The activity of enzyme
A was doubled when the ionic strength was increased from
0.012 to 0.20, while there was no effect of ionic strength on
the activity of enzyme B.

**Temperature Stability.** Temperature stabilities of pear en-
zymes A and B are shown in Figure 4. The two enzymes have
similar heat stability characteristics except for the 10-min lag

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme A</td>
</tr>
<tr>
<td></td>
<td>( \mu )mol O(_2)/min-mg protein</td>
</tr>
<tr>
<td>0.012</td>
<td>6.95</td>
</tr>
<tr>
<td>0.10</td>
<td>10.0</td>
</tr>
<tr>
<td>0.20</td>
<td>12.0</td>
</tr>
<tr>
<td>0.30</td>
<td>13.6</td>
</tr>
<tr>
<td>0.40</td>
<td>14.2</td>
</tr>
<tr>
<td>0.50</td>
<td>14.4</td>
</tr>
</tbody>
</table>

**Fig. 4.** Heat stabilities of pear enzymes A and B. Enzyme A
(50 \( \mu \)g/ml) and enzyme B (31 \( \mu \)g/ml) in 0.1 M phosphate, pH 6.2,
were heated in sealed tubes at various times at either 50 C (\( \circ \): en-
zyme A; \( \bullet \): enzyme B) or 75 C (\( \Delta \): enzyme A; \( \Delta \): enzyme B).
Remaining activity was determined with an oxygen electrode at
pH 4.0 and 30 C with chlorogenic acid as substrate.
in loss of activity of enzyme A at 75 C. Both enzymes lost no activity over a period of 60 min when incubated at 50 C, pH 6.2, and a protein concentration of 50 and 31 µg/ml for enzymes A and B, respectively. After heating for 60 min at 75 C, 35 and 27% of the activity of enzymes A and B, respectively, remained. Relatively high heat stabilities are found for most polyphenol oxidases.

**Effect of Oxygen Concentration.** Polyphenol oxidase-catalyzed reactions are two substrate reactions with oxygen as the second substrate. The effect of systematically varying each substrate concentration can give valuable information about the mechanism of action of polyphenol oxidase.

The effect of chlorogenic acid and oxygen concentrations on the initial velocity of enzyme B-catalyzed oxidation of chlorogenic acid is shown in Figure 5. Reciprocal plots of substrate concentration versus initial velocity give a series of lines which intersect to the left of the vertical axis. Therefore, the experimental results suggest a sequential mechanism (23) for the binding of chlorogenic acid and oxygen to pear polyphenol oxidase. Gregory and Bendall (5) found that with tea polyphenol oxidase series of parallel lines (indicative of a Ping Pong mechanism [23]) were obtained for most substrates at different oxygen concentrations. The exception was chlorogenic acid, which gave a series of lines converging at the vertical axis. With mushroom polyphenol oxidase, a series of parallel or very slightly convergent lines was obtained by pyrocatechol, whereas series of convergent lines were obtained in the case of acetyl catechol and 4-methyl catechol (4). There is no good explanation of why the plots should be different with different substrates.

In a sequential mechanism, one of the substrates may be required to bind to the enzyme before the second substrate can bind (ordered mechanism) or the two substrates may bind randomly (random mechanism) to the enzyme (23). Ingraham (8) in a study on the mechanism of a polyphenol oxidase from French prunes found his data were consistent with a mechanism in which oxygen bound first to the enzyme. Duckworth and Coleman (4) reported that, if the mechanism of mushroom polyphenol oxidase is ordered, oxygen does not bind first. The data with tea polyphenol oxidase are consistent with a random mechanism (5). Why such divergent results are found by different workers on different polyphenol oxidases must await additional data on these enzymes. We can make no conclusions about the order of addition of substrates to the pear polyphenol oxidases from our data.

The following values for the kinetic constants were calculated from the data of Figure 5: $V_{max}$ of 103 µmoles of oxygen uptake/min·mg enzyme, $K_m$ for oxygen of 0.11 mm and $K_m$ for chlorogenic acid of 7.2 mm at pH 4.0. Thus, under atmospheric oxygen concentrations of 0.24 mm, the enzyme is about 68% saturated with oxygen.

**Effect of pH on Activity.** The effect of pH on $V_{max}$ and $K_m$ for oxidation of chlorogenic acid by enzymes A and B is shown in Figures 6 and 7 and Table IV. At each pH the initial velocity was determined as a function of chlorogenic acid concentration (Fig. 6). The series of lines of the reciprocal plots con-
verge on the X-axis to the left of the vertical axis. Thus, the hydrogen ion is a simple, noncompetitive inhibitor (23) of the polyphenol oxidases, and Km is independent of hydrogen ion concentration (Table IV).

The Vmax values are quite dependent on pH (Table IV, Fig. 7). In Figure 7, the Vmax values, relative to the value at pH 4.0, are plotted as a function of pH. The pH-Vmax profiles for both enzymes are probably bell-shaped with similar dependencies on pH on the acid side of the pH optimum, but they show differences in their pH dependencies on the alkaline side. The best values for the pK values are: 3.5 ± 0.2 and 5.2 ± 0.5 for enzyme A, and 3.2 ± 0.2 and 6.7 ± 0.5 for enzyme B. With enzyme B, the pH activity data do not exclude the possibility of two pH optima.

Widely different pH optima have been reported for various plant polyphenol oxidases. The pH optima of tea polyphenol oxidase on pyrogallol and 4-methyl catechol were found to be 5.7 and 5.0, respectively (5). Banana polyphenol oxidase had a pH optimum of 7 on dopamine (13). pH optimum for oxidation of chlorogenic acid by potato polyphenol oxidase was 4.3 (1), whereas that for apple polyphenol oxidase was 5 (21). The pH optima for clingstone peach polyphenol oxidases were 6.5 to 7.2 on catechol (24). In many of the cases, the relationship between substrate concentration and Km as function of pH is not known. It is of interest that the pH of Bartlett pears at canning ripeness is pH 4 (18).

The polyphenol oxidases were stable for 2 hr at 30 C and pH 7. Between pH 4 and 7 there was about 20% loss in activity under these conditions, while the activity loss was 75 and 97% at pH 3.5 and 3.0, respectively, after 2 hr. Since initial rates were used in these studies on effect of pH on activity, enzyme instability is probably not responsible for the effect of pH on the acid side of the pH optimum and certainly not on the alkaline side.

The data indicate that the prototropic groups of pK 3.5 and 5.2 for enzyme A and pK 3.2 and 6.7 for enzyme B are involved in oxidation of chlorogenic acid and not in its binding to enzyme (pH independence of Km for chlorogenic acid). It is not possible to assign specific groups to these pK values at this time.

**Inhibition of Enzymes A and B.** Inhibition of pear enzymes A and B by p-coumaric and benzoic acids are shown in Figure 8. With p-coumaric acid, the series of lines of the reciprocal

### Table IV. Michaelis Parameters for Pear Polyphenol Oxidase Oxidation of Chlorogenic Acid as Function of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Enzyme A</th>
<th>Enzyme B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (mM)</td>
<td>Vmax (μmoles O2/min·mg protein)</td>
</tr>
<tr>
<td>3.0</td>
<td>9.50 ± 1.87</td>
<td>12.9 ± 2.1</td>
</tr>
<tr>
<td>3.5</td>
<td>9.10 ± 3.71</td>
<td>24.9 ± 5.4</td>
</tr>
<tr>
<td>4.0</td>
<td>11.7 ± 3.2</td>
<td>42.6 ± 10.4</td>
</tr>
<tr>
<td>5.0</td>
<td>11.4 ± 3.5</td>
<td>22.8 ± 6.1</td>
</tr>
<tr>
<td>6.0</td>
<td>8.30 ± 0.64</td>
<td>13.6 ± 5.6</td>
</tr>
<tr>
<td>7.0</td>
<td>8.20 ± 1.38</td>
<td>14.4 ± 5.9</td>
</tr>
</tbody>
</table>

**Fig. 8.** Inhibition of pear enzyme A- and enzyme B-catalyzed oxidation of chlorogenic acid by p-coumaric and benzoic acids. The reactions were followed with an oxygen electrode at 30.0 C and pH 6.2 (p-coumaric acid) or pH 4.0 (benzoic acid). The buffers were 25 mM succinate-25 mM pyrophosphate with ionic strength adjusted to 0.22 with NaNO3. Activities are expressed per ml of enzyme which contained 50.0 and 31.0 μg/ml of protein for enzymes A and B, respectively. Chlorogenic acid concentrations ranged from 1 to 5 mM. Concentrations of p-coumaric acid were: ○, no inhibitor; □, 0.1 and 0.2 mM for enzymes A and B, respectively; △, 0.4 mM; X, 0.75 mM. Concentrations of benzoic acid were: ○, no inhibitor; □, 0.1 mM; △, 0.25 mM; X, 0.366 mM.

plot converge on the X-axis to the left of the vertical axis. Replots of the slopes and intercepts as a function of inhibitor concentration gave straight lines. Thus, p-coumaric acid gives simple, linear noncompetitive inhibition (23) of both enzymes which indicates that the substrate, chlorogenic acid, and p-coumaric acid bind at different sites on the enzyme. Km values for p-coumaric acid were 0.38 and 0.50 mM for enzymes A and B, respectively.

A number of monophenols are inhibitory of polyphenol oxidase activities (12, 15). p-Coumaric acid was found to be a noncompetitive inhibitor of potato tuber polyphenol oxidase-catalyzed oxidation of chlorogenic acid (12) with Km of 5.2 mM; however, the series of lines of the reciprocal plot converged above the X-axis.

Inhibition of pear enzymes A and B by benzoic acid, with chlorogenic acid as substrate, gave a series of lines which converged on the Y-axis. Plots of slopes of the lines as a function of benzoic acid concentration gave straight lines. Thus, benzoic acid is a linear competitive inhibitor (23) of these enzymes which indicates that it and chlorogenic acid bind at the same site or overlapping sites on the enzymes. The Km values were 0.04 and 0.11 mM for enzymes A and B, respectively, based on the unionized benzoic acid concentration at pH 4.0 (15).

Benzoic acid has been found to be a competitive inhibitor of other polyphenol oxidases (4, 10, 13). The Km value for benzoic acid inhibition of mushroom polyphenol oxidase-catalyzed oxidation of catechol was reported to be 1.02 μM (4); this value is much lower than the values found for pear polyphenol oxidases and most other polyphenol oxidases.

**LITERATURE CITED**

POLYPHENOL OXIDASES OF BARTLETT Pears


