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Isolation and Characterization of Polyphenol Oxidase Isozymes of Clingstone Peach[1]

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ABSTRACT

The polyphenol oxidase system in clingstone peach (*Prunus persica*) was investigated. Polyacrylamide disc-gel electrophoresis indicated four bands with polyphenol oxidase activity in extracts from acetone powder of clingstone peach. These four isozymes were then isolated from a buffer extract of peach acetone powder by cold acetone precipitation, followed by diethylaminoethyl cellulose column chromatography. All isozymes had different heat stabilities. At 55 C, polyphenol oxidases A, B, and D had half-lives of 5.4, 14.6, and 14.1 minutes, respectively. Polyphenol oxidase C was stable over a period of 50 minutes of incubation at 55 C, but had a half-life of 2.2 minutes at 76 C. None of the isozymes had monophenolase activity, and they varied in their specificity for several diphenols. The following values were found for polyphenol oxidases A, B, C, and D, respectively, with catechol as substrate: optimal pH: 6.8, 6.5, 7.2, and 7.0; Michaelis constant: 6.6, 4.2, 7.0, and 36 mM; $V_{max}/(E_0)$: 4.95, 39.4, 2.16, and 80.0 (ΔA min^{-1} mg^{-1}). Each isozyme showed a different amount of inhibition by $NaHSO_3$, NaCl, NaCN, L-ascorbic acid, glutathione, ethylenediaminetetraacetate, and sodium diethyldithiocarbamate.

Polyphenol oxidase (PPO[3]; *o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1; also known as phenolase, phenol oxidase, catechol oxidase, and tyrosinase) is widely distributed in nature. Multiple forms of PPO have been isolated from a wide variety of sources, including mushroom (1, 3, 10, 11, 20), apple chloroplasts (8, 9), broad bean (19), potato (3, 17), *Neurospora* (5, 6), and melanoma (18). The reported number of multiple forms (isozymes) of PPO range from 2 in melanoma to as many as 11 in potato (3).

The two PPO isozymes from hamster melanoma were found to be similar with respect to their pH optima, sedimentation, behavior toward several inhibitors, Michaelis constants, and maximal velocities (18). However, they were different in electrophoretic and chromatographic properties. Some of the isozymes of PPO from mushrooms are high in cresolase activity while others are high in catecholase activity (20). The mushroom PPO system was reported to consist of nine DL-DOPA-reactive PPOs, but only three of these PPOs were found to have activity toward L-tyrosine (3). Peaches were reported to contain at least three PPO isozymes with activity toward DL-DOPA (3).

In the present work, four PPO isozymes were isolated from clingstone peach by using DEAE-cellulose column chromatography. Their characteristics with respect to pH optima, substrate specificity, kinetic properties, as well as behavior toward several inhibitors, are reported here.

MATERIALS AND METHODS

Peaches. Clingstone peach (*Prunus persica* var. Cortez) at canning ripeness was obtained from an orchard in Wheatland, California. About 150 pounds of peaches were pitted, halved, quick frozen in Freon 12 and sealed in No. 2½ cans under a vacuum of 16 inches of Hg. The samples were stored at −26 C until used.

All purification steps were carried out at 1.1 C and all reagents were prepared in deionized water.

Preparation of Acetone Powder. After thawing at 1.1 C for 4 hr, 1 kg of peach was homogenized in a large Waring Blendor with 2 liters of cold acetone (−26 C) and 6.7 g of polyethylene glycol (mol. wt. 6,000–7,000, J. T. Baker Chemical Co.) for 1 min. The yellow slurry was filtered under suction. The above step was repeated on the solids without polyethylene glycol. The wet cake was washed repeatedly with cold acetone until most of the pigments were removed. The material was allowed to dry at room temperature overnight to give about 44 g total weight of powder.

Isolation of Polyphenol Oxidase Isozymes. Peach acetone powder (44 g) was stirred mechanically with 880 ml of 0.05 M phosphate buffer at pH 6.2 for 30 min. The slurry was centrifuged at 4,080*g* and 0 C for 20 min. The supernatant fraction was recentrifuged at 73,400*g* for 30 min to give 620 ml of a rice-colored, viscous, but nearly clear liquid.

The enzyme in the supernatant fraction was precipitated by slow addition of 2 volumes of cold (−25 C) acetone. After 5 min, the major portion of the liquid was decanted and discarded. The white precipitate was collected by centrifugation at 4,080*g* for 30 min and resuspended in a minimal amount (about 250 ml) of 0.01 M phosphate buffer at pH 6.2. After approximately 2 hr of continuous stirring, a viscous and gel-like suspension was obtained.

To remove the pectic substances, calcium acetate was added to the viscous suspension to a final concentration of 1.1 M. The pH was adjusted to 6.8 with dilute NaOH. The suspension became more viscous on standing in a refrigerator overnight, but no liquid separated on standing. During subsequent dialysis against four changes of 3 liters of 1×10^{-3} M phosphate buffer,

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[3] Abbreviations: PPO: polyphenol oxidase; DOPA: dihydroxyphenylalanine; DEAE: diethylaminoethyl.

pH 6.2, a clear, light yellow liquid separated, and the gel became firmer. The suspension was centrifuged at 27,000*g* for 30 min, and the precipitate was washed with an equal volume of 0.24 M calcium acetate solution, pH 6.2. The washing was added to the supernatant fraction, and the precipitate was discarded.

The supernatant fraction was dialyzed again for 24 hr against two changes of 1×10^{-3} M phosphate buffer, pH 6.2. During dialysis a thin gel separated and was removed by centrifugation at 27,000*g* for 30 min. The solution was then perfectly clear and had a viscosity similar to that of water.

The enzyme solution was concentrated to 49 ml by ultrafiltration (Diaflo membrane, UM10, 76 mm size, Amicon Corp., Lexington, Mass.) under a pressure of 80 p.s.i. for further purification by DEAE-cellulose column chromatography.

DEAE-cellulose of medium mesh, 1.0 meq/g, was obtained from Sigma Chemical Co., St. Louis, Missouri. Before use, 50 g of DEAE-cellulose were washed with 500 ml of 0.25 M NaOH-0.25 M NaCl and then with 500 ml of deionized water. The latter step was repeated four times. It was then washed once with 500 ml of 0.25 M HCl and suspended in a 1-liter graduated cylinder filled with deionized water for removal of fine particles. After removal of fine particles and adjusting the pH to 6.2, the DEAE-cellulose was washed five times with 1×10^{-3} M phosphate buffer at pH 6.2.

The column bed used was 1 cm in diameter and 45 cm in length. The column was equilibrated with 250 ml of 1×10^{-3} M phosphate buffer, pH 6.2, in a cold room (1.1 C) before use.

After addition of 49 ml of enzyme solution, the column was eluted with two sequential linear gradients as described in Figure 2.

Fractions under the first peak from the DEAE-cellulose chromatographic separation (Fig. 2) were pooled, concentrated by ultrafiltration, and dialyzed against two changes of 7.5×10^{-4} M phosphate buffer at pH 6.2. A second DEAE-cellulose column was prepared in the same way as above except it was equilibrated with 7.5×10^{-4} M phosphate buffer, pH 6.2. After application of the concentrated enzyme solution (10 ml) from peak 1, the column was eluted with a linear gradient from 7.5×10^{-4} M phosphate (125 ml) to 1×10^{-2} M NaCl in 5×10^{-2} M phosphate (125 ml), pH 6.2.

Electrophoresis. Polyacrylamide gel electrophoresis was performed according to Davis (4) and Whitaker (21). For preparation of crude enzyme, approximately 1 g of peach acetone powder was stirred with 20 ml of 0.05 M phosphate buffer, pH 6.2, for 30 min. The suspension was centrifuged at 27,000*g* for 20 min in a Sorvall refrigerated centrifuge at 0 C and recentrifuged at 73,400*g* for 30 min in a model L Spinco ultracentrifuge at 0 C.

About 0.1 ml of sample (1.82 mg/ml of protein) was mixed with 0.2 ml of 2% sucrose for each analysis. The gel tube was 0.5 × 8.9 cm long and contained 5.0 cm of running gel (7%) and 1 cm of 1.25% spacer gel. The sample was introduced into the top 2.9-cm length of the tube. The starting pH was 8.3 and the running pH was 9.5. A current of 2.0 ma per tube was employed at 1.1 C until bromophenol blue, used as a reference marker, migrated close to the end of the tube (about 2 hr).

Part of the gels were developed by staining with 1% Amido Black 10B (K & K Laboratories, Jamaica, N. Y.) in 7% acetic acid for 15 min, followed by washing with 7% acetic acid for at least 24 hr. The other gels were washed thoroughly with 0.1 M citrate-0.2 M phosphate buffer, pH 6.0, and then allowed to react with enzyme substrates, catechol, *d*-catechin, and gallic acid, all at a concentration of 0.03 M and pH 6.0 in the same buffer. The gels were stored in 30% ethyl alcohol.

Protein Estimation. The concentration of protein was determined by a modification (15) of the method of Lowry *et al.* (13) with crystalline bovine serum albumin (Armour Pharmaceutical Company, Oakbrook, Ill.) as a standard.

Enzyme Activity Measurements. PPO activity was determined by measuring the initial rate of quinone formation as indicated by an increase in absorbance at 420 nm (12). A Beckman DB spectrophotometer was employed throughout the investigation. One unit of enzyme activity is defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. Because of rapid product inhibition of the enzyme, only the initial increase in absorbance (first 30 sec after a reaction had been started) was taken as the rate of a reaction.

Unless otherwise specified, a buffer of 0.1 M citrate-0.2 M phosphate at pH 6.8 was used in all reactions, and the reactions were carried out at 30.0 ± 0.5 C. In all cases, the initial rate of reaction was proportional to concentration of enzyme.

Heat Stability. Protein concentrations of all four PPO isozymes were adjusted to 0.08 mg/ml and pH 6.2. Five milliliters of each isozyme solution were pipetted into a prewarmed test tube in a water bath at 55.0 ± 0.2 C. A 0.5-ml aliquot of the heated enzyme solution was withdrawn at appropriate times and immediately assayed for remaining activity at 30 C in a cuvette containing 2.5 ml of 10 mM catechol. The heat stability of PPO C was also tested at 76.0 ± 0.2 C.

Substrate Specificity. All substrates were commercial grade and were used without further purification. For activity assays, 0.2 ml of enzyme solution was stirred rapidly into 2.8 ml of 10 mM substrate dissolved in 0.1 M citrate-0.2 M phosphate buffer, pH 6.8, in the reaction cuvette. The rate of reaction was recorded continuously at 420 nm.

pH Optima. Enzyme activity as a function of pH was determined with 10 mM catechol in 0.1 M citrate-0.2 M phosphate buffers ranging from pH 4 to 8.

Effect of Substrate Concentration. Concentrations of catechol ranging from 2.8 to 28 mM were used for this study. To initiate reaction, 0.2 ml of enzyme solution was mixed rapidly with 2.8 ml of a solution of catechol in 0.1 M citrate-0.2 M phosphate buffer, pH 6.8 and 30 C. The reaction was followed at 420 nm. Michaelis constants and maximum velocities were calculated by least squares treatment of data plotted as 1/activity *versus* 1/(substrate).

Inhibitor Studies. To determine the effect of inhibitors, reactions containing 9 mM catechol and a constant amount of enzyme in 0.1 M citrate-0.2 M phosphate, pH 6.8, were run at 30 C in the presence and absence of the inhibitor. The reaction was followed continuously at 420 nm, and rate of reaction was determined from the slope of the reaction curve following any delay in change in absorbance at 420 nm due to the inhibitor.

RESULTS

Electrophoresis. Four polyphenol oxidase isozymes with catechol and *d*-catechin activities were separated by polyacrylamide gel electrophoresis (Fig. 1). Only three bands with gallic acid activity were observed. The substrate specificity studies confirmed that PPO D did not have activity toward gallic acid.

Purification of Peach Polyphenol Oxidase. A thick, gel-like material resulted from suspension of the acetone precipitate in 1×10^{-2} M phosphate buffer, pH 6.2. No further purification could be performed until the pectic substances were removed. Attempts, such as digestion by pectinase, adsorbing the enzyme by passing the diluted gel solution through a layer of DEAE-cellulose, and precipitating the enzyme with various concentrations of ammonium sulfate or cold acetone, all failed to give satisfactory removal of the pectic material. Ad-

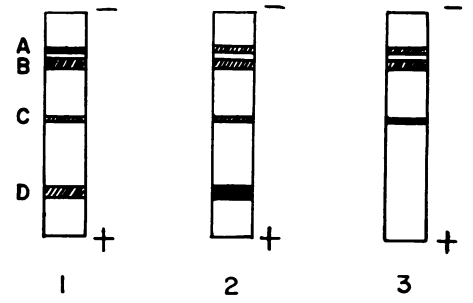

FIG. 1. Polyacrylamide disc-gel electrophoresis of buffer extract from clingstone peach acetone powder. Spacer gel, 1.25%, running gel, 7%; stacking was at pH 8.3, running at pH 9.5. After electrophoresis, gel patterns were developed with 0.03 M catechol (1), 0.03 M *d*-catechin (2), and 0.03 M gallic acid (3), all in 0.1 M citrate-0.2 M phosphate buffer, pH 6.0. The origin is at the top edge of the gel.

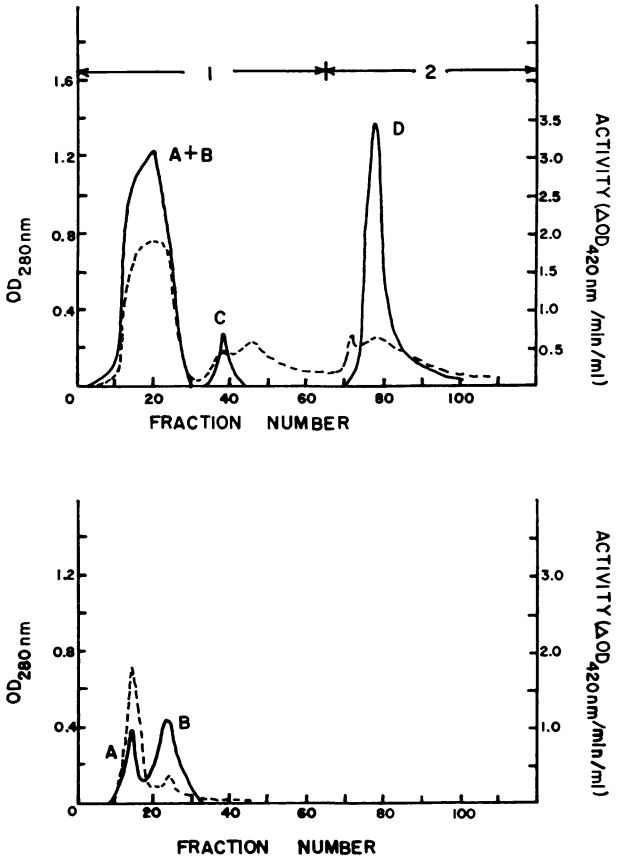

FIG. 2. DEAE-cellulose chromatographic pattern of peach polyphenol oxidases. Protein concentration (dotted line) was estimated at 280 nm. Enzyme activity (solid line) was assayed in the same way as indicated in Table I. Three-milliliter fractions were collected. The linear buffer systems used to elute the protein were: (upper) 1, 100 ml of 1×10^{-3} M phosphate-100 ml of 1×10^{-2} M NaCl in 5×10^{-2} M phosphate; 2, 100 ml of 1×10^{-2} M NaCl in 5×10^{-2} M phosphate-100 ml of 0.25 M phosphate; (lower) 125 ml of 7.5×10^{-4} M phosphate-125 ml of 1×10^{-2} M NaCl in 5×10^{-2} M phosphate; all were at pH 6.2.

dition of calcium acetate and formation of a firmer gel did not cause separation of liquid from the gel. However, dialysis of the gel against phosphate buffer after addition of calcium acetate gave a clear enzyme solution which could be fractionated on DEAE-cellulose.

Three peaks with PPO activity were obtained by DEAE-cellulose chromatography (Fig. 2). Rechromatography of the first peak gave two partially separated activity peaks. Additional modifications in the chromatographic procedure did not result in better separation. The overlapping fractions which contained both PPO A and B were discarded. Polyacrylamide gel electrophoresis indicated that each isozyme fraction contained at least three to four other rather minor protein components. However, except for PPO B, which contained a small amount of PPO A, only one catechol-active band was observed for each isozyme.

The designations A, B, C, and D used for each isozyme according to the order of elution from the DEAE-cellulose column corresponded to the order of migration on electrophoresis. Table I summarizes the purification of polyphenol oxidase from 1 kg of clingstone peach.

Heat Stability. Although PPO A and B behaved similarly in their elution from a DEAE-cellulose column, they had different heat stabilities. The half-lives of PPO A and B at 55 C were 5.4 and 14.6 min, respectively (Fig. 3). Under the same conditions PPO D had a half-life of 14.1 min. The most striking difference among the four isozymes was the stability of PPO C. PPO C was stable over a period of 50 min of incubation at 55 C, although it was inactivated rapidly at 76 C ($t_{1/2}$ = 2.2 min).

Substrate Specificity. A number of *o*-diphenols and some monophenols and *m*-diphenols were tested in a study of substrate specificity of the isozymes. All the isozymes had activity toward *o*-diphenols (Table II), but they had no activity toward monophenols and *m*-diphenols even in the presence of a small amount of the *o*-diphenol, catechol.

The monophenolase activity of a PPO is generally more unstable than the *o*-diphenolase activity (14). To test if monophenolase activity were lost during purification, its activity was assayed using a sample from carefully pressed peach juice. No monophenolase activity was found with or without a small amount of catechol.

PPO D did not have activity toward protocatechuic and gallic acids, and PPO A and B did not have activity toward caffeic acid under the conditions used. The data in Table II should be used only as an indication of relative differences among the four isozymes since the degree of saturation of each isozyme with a particular substrate is not known. Furthermore, comparisons of specific activities among the different substrates is only approximate, as extinction coefficients of the quinones produced are not identical at 420 nm.

pH Optima. pH optima were 6.8, 6.5, 7.2, and 7.0 for PPO

Table I. *Purification of Polyphenol Oxidase from Peach*
One kilogram of peaches was used as starting material.

Procedure	Volume	Activity[1]		Protein Concn	Total Protein	Specific Activity
	ml	*units/ml*	*units* $\times 10^{-4}$	*mg/ml*	*mg*	*units/mg* $\times 10^{-4}$
1. Buffer extract	620	725	45	1.24	769	0.6
2. Acetone precipitate	49	5400	26.5	3.5	169	1.6
3. DEAE-cellulose chromatography						
A	20.5	1275	2.6	0.8	16.4	1.6
B	37	890	3.3	0.08	2.96	11.0
C	20	425	0.85	0.32	6.4	1.3
D	83	850	7.06	0.3	24.9	2.8
Total			13.81		50.66	

[1] One unit = ΔA of 0.001 at 420 nm per min. The enzyme activity was assayed in a cuvette by using 2.8 ml of 0.01 M catechol in 0.1 M citrate-0.2 M phosphate buffer, pH 6.2, and 0.2 ml of enzyme solution at 30 C.

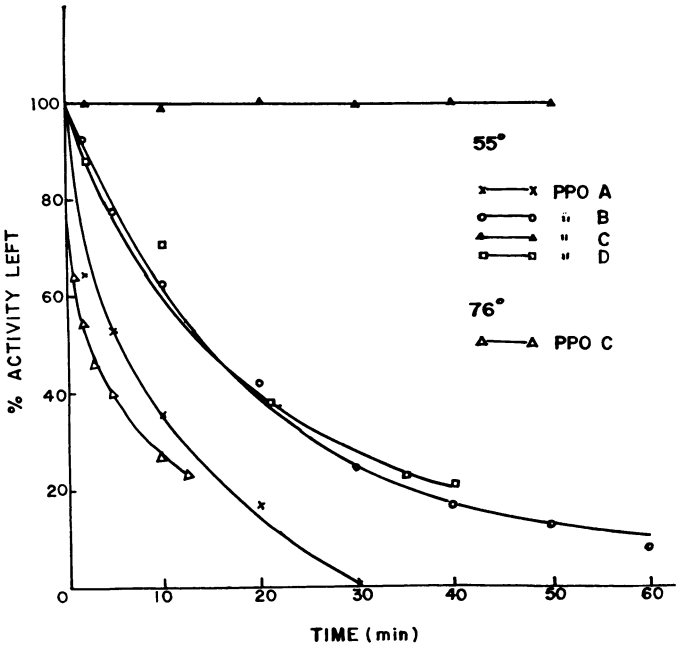

Fig. 3. Heat stabilities of peach polyphenol oxidase isozymes. After various lengths of time at the indicated temperature, aliquots were removed for activity assay as described in Table I. Protein concentration for all isozymes was 0.08 mg/ml.

Table II. *Substrate Specificity of Peach Polyphenol Oxidases on o-Diphenols*

Activity was determined as the initial rate of change in absorbance at 420 nm and 30 C. The substrate concentration was 9.3 mM. The reaction mixture contained 0.1 M citrate-0.2 M phosphate buffer, pH 6.8.

Substrate[1]	Specific Activity			
	A	B	C	D
	units/mg protein			
Catechol	1070	9380	1120	2270
d-Catechin	340	6880	2500	3000
Chlorogenic acid	238	2500	390	1830
Dopamine	488	3750	234	747
Protocatechuic acid	175	81	470	0
Caffeic acid	0	0	438	31
DOPA	432	3750	688	600
Gallic acid	275	1050	1300	0
4-Methylcatechol	550	3130	1410	1830

[1] No activity was obtained with phenol, tyrosine, *p*-coumaric acid, *p*-cresol, ferulic acid, phloroglucinol, resorcinol, or orcinol.

A, B, C, and D, respectively. The pH-activity profiles for all isozymes were bell-shaped curves, and there was little or no activity for any of the isozymes below pH 5 and above 8.

Effect of Substrate Concentration. The effect of catechol concentrations ranging from 2.8 to 28 mM on the initial rate of reaction was determined for each isozyme. In all cases, straight lines were obtained for the Lineweaver-Burk plots. Michaelis constants and maximal velocities for the isozymes were determined from the data by use of a least squares treatment (Table III). PPO A, B, and C had similar K*m* values for catechol while that of PPO D was 5 to 9 times larger than for the other three isozymes. PPO A and C had similar maximal velocities, which were several fold lower than the maximal velocities for PPO B and D.

At 30 C, rapid initial stirring of the reaction mixture provided a sufficiently high concentration of oxygen so that it was not a limiting factor in the oxidation of catechol, since increases in oxygen concentration by bubbling oxygen at different rates through the reaction mixture did not increase the rate of reaction. Therefore, oxygen is apparently present in the reaction mixture at 30 C during the first 30 sec of reaction at a sufficient concentration to saturate the enzyme.

Inhibitor Studies. $NaHSO_3$, ascorbic acid, glutathione, and sodium diethyldithiocarbamate all caused a delay in time until there was an increase in absorbance at 420 nm. The percentage inhibition by the various compounds was calculated from the rate of change in absorbance following this delay time (Table IV).

The four isozymes showed quite different amounts of inhibition by the various compounds. For example, PPO A was not inhibited at all by the highest concentrations of $NaHSO_3$, ascorbic acid, and glutathione used while PPO D was quite sensitive to lower concentrations of ascorbic acid and glutathione. All four isozymes were inhibited by NaCN; however, PPO C was much more sensitive to this compound. Compared to the

Table III. *Some Characteristics of Peach Polyphenol Oxidase Isozymes with Catechol as Substrate*

Catechol concentrations ranged from 2.8 to 28 mM. The buffer was 0.1 M citrate-0.2 M phosphate, pH 6.8, and the temperature was 30.0 C.

PPO	K*m*	k_0[1]	Relative k_0
	mM	*ΔA/min·mg protein*	
A	6.6	4.95	2.3
B	4.2	39.4	18.3
C	7.0	2.16	1.0
D	36.0	80.0	37.0

[1] $V_{max}/(E_0)$ where (E_0) is in mg/ml.

Table IV. *Effect of Inhibitors on Activity of Polyphenol Oxidases A, B, C, and D*

Substrate concentration was 9.0 mM catechol. The buffer was 0.1 M citrate-0.2 M phosphate, pH 6.8, and the temperature was 30.0 C.

Inhibitor	Inhibition				Inhibitor	Inhibition			
	A[1]	B	C	D		A	B	C	D
	%	%	%	%		%	%	%	%
NaCl (M)					Ascorbic acid (μM)				
0.2	10	0		74	33.0		0	43	88
0.5	31	33	37	88	66.0	0	26	59	96
0.8	48	47	55	93	99.0		41	77	
NaCN (μM)					132	0			
9.9			37		198	0			
13.2			77		Glutathione (μM)				
16.5			86		33.0	0	0	0	73
33.0	32	33		14	66.0	0	2	22	84
99.0	56	51		29	EDTA (M)				
165	84	70		40	0.1	33	60	81	35
$NaHSO_3$ (μM)					Sodium diethyldithiocarbamate (μM)				
33.0		10	20	21					
66.0	0	64	50	27	33.0	23	28	11	57
165	0			65	66.0	31	40	36	84
231	0								

[1] Concentrations (activities) of enzyme were: A, 53.4 μg/ml (57.1 units/ml); B, 5.34 μg/ml (50.2 units/ml); C, 21.3 μg/ml (23.9 units/ml); D, 20.0 μg/ml (45.4 units/ml).

other compounds, NaCl and EDTA were not very effective inhibitors. Why there should be such a marked difference among the isozymes in their response to these compounds must await further study.

DISCUSSION

Based on heat stabilities, pH optima, substrate specificity, kinetic and inhibitor studies, and electrophoretic and chromatographic behavior, the four isozymes of PPO isolated from clingstone peach were found to be different. PPO A and B were similar in many aspects, such as chromatographic and electrophoretic properties, substrate specificity, and Michaelis and reaction rate constants, but they were markedly different in their stabilities at 55 C.

All four isozymes were found to be *o*-diphenolases. Their inability to catalyze the oxidation of monophenolic compounds is in contrast to some other polyphenol oxidases, such as those from mushroom and potato, which possess both monophenolase and *o*-diphenolase activities. No monophenolase activity was found in fresh peach juice either. Polyphenol oxidases that have only *o*-diphenolase activity have also been isolated by other investigators from tobacco leaf (2), tea leaf (7), and banana (16).

In the inhibitor studies, $NaHSO_3$, L-ascorbic acid, and glutathione delayed the increase in absorbance at 420 nm, presumably because of their ability to reduce *o*-benzoquinone back to catechol as it was formed. The length of this delay period depended on the concentrations of reducing agent used, which is expected. These reducing agents also inhibited PPO B, C, and D. However, PPO A was not inhibited by these compounds. It is difficult to explain the resistance of PPO A to these inhibitors.

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