Partial Characterization of Polyphenol Oxidase Activity in Raspberry Fruits

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A partial characterization of polyphenol oxidase (PPO) activity in raspberry fruits is described. Two early cultivars harvested in May/June (Heritage and Autumm Bliss) and two late cultivars harvested in October−November (Ceva and Rubi) were analyzed for PPO activity. Stable and highly active PPO extracts were obtained using insoluble poly(vinylpyrrolidone) (PVP) and Triton X-100 in sodium phosphate, pH 7.0 buffer. Polyacrylamide gel electrophoresis of raspberry extracts under nondenaturing conditions resolved in one band ($R_{f1} = 0.25$). Raspberry PPO activity has pH optima of 8.0 and 5.5, both with catechol (0.1 M). Maximum activity was with D-catechin (catecholase activity), followed by *p*-coumaric acid (cresolase activity). Heritage raspberry also showed PPO activity toward 4-methylcatechol. Ceva and Autumm Bliss raspberries showed the higher PPO activity using catechol as substrate.

Keywords: *Raspberry; polyphenol oxidase; characterization; native-PAGE*

INTRODUCTION

Raspberries are a very perishable commodity, partly due to high respiration and transpiration rates, a soft texture that predisposes them to crushing, and susceptibility to gray mold fruit rot. Techniques providing even a short extension of shelf life could have a profound effect on fresh-marketing raspberries. On the basis of respiratory quotient, ethanol production, and flavor, Joles et al. (1994) recommended that raspberries be stored at oxygen levels above 4 kPa at 0 °C, 6 kPa at 10 °C, and 8 kPa at 20 °C. However, these authors also reported that fermentative conditions could develop if perforated packages experienced an increase in temperature, because beneficial CO_2 levels (∼20 kPa) cannot be reached without inducing fermentation. In all cases, prompt and continuous cooling of the harvested raspberries to 0 °C is essential to preserve their marketable life for 2−3 days in air. Holding conditions of 0 °C and 90−95% relative humidity throughout their postharvest handling stages have been recommended (Robbins and Fellman, 1993).

Frozen fruit can be transported to remote markets that could not be accessed with fresh fruit. Freezing also makes year-around further processing of fruit products (such as jams, juice, and syrups from frozen raspberries) possible (Skrede, 1996). Factors such as cultivar, maturity, growing area, and seasonal variations influence frozen processing performance of fruits to an extent that may override the positive effect of a quick freezing rate. Bushway et al. (1992) evaluated five raspberry cultivars harvested at the red-ripe stage at harvest and during frozen storage at −20 °C. In this study, a significant decrease in shear values (firmness) for all raspberry cultivars during 9 months of storage was observed.

The presence of polyphenol oxidase (monophenol dihydroxyphenylalanine:oxygen oxidoreductase: E.C. 1.14.18.1; PPO) in fruit and vegetable tissues is of concern to food processors and researchers. PPO catalyzes formation of highly active quinones that react with amino or sulfhydryl groups in proteins or enzymes. These reactions lead to changes in physical, chemical or nutritional characteristics of proteins and, in many cases, to inactivation of enzymes including PPO (Mayer and Harel, 1979). Quinones also lead to polymerization and condensation reactions between proteins and polyphenols forming brown pigments (Lee, 1991; Mathew and Parpia, 1971). PPO activity may also be responsible for loss of red color of some fruits by degrading anthocyanin pigments (Markakis, 1974).

PPO activity in other berry fruits as strawberry (Wesche-Ebeling and Montgomery, 1990a,b) and blueberry (Kader et al., 1997) had been reported. However, little is known about the PPO in raspberry fruits and its involvement in anthocyanin degradation. The purpose of the present study was to develop a procedure for the extraction of PPO from raspberry fruit tissues, its partial characterization, and the variation in its activity among raspberry cultivars in terms of PPO enzyme.

MATERIALS AND METHODS

Plant Material. Raspberry fruits (*Rubus idaeus* L.) of four cultivars (cvs. Autumm Bliss, Heritage, Rubi, and Ceva), were obtained form commercial producers in the region of Valle del Jerte (Cáceres, Spain) and transported under refrigeration to Instituto del Frío (Madrid, Spain) within 12 h after harvest. On arrival, undamaged fruits were selected with the characteristics shown in Table 1. One kilogram of each cultivar was homogeneized using a blender (Osterizer) and the pulps obtained were used immediately for physical and physicochemical determinations. Another 2 kg of each cultivar was immediately frozen in liquid nitrogen and stored at −70 °C until biochemical analysis was performed.

pH and Titratable Acidity. A 10 g portion of raspberry pulp was minced and blended with 40 mL of deionized water in a Sorvall Omnimixer. The pH was measured at this

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Table 1. Physicochemical Characteristics and PPO Activities of Spanish Raspberry Cultivars

characteristics[a]	raspberry cultivars			
	Autumm Bliss	Heritage	Ceva	Rubi
pH	3.65 ± 0.10[a]	3.87 ± 0.02[b]	2.88 ± 0.02[c]	2.65 ± 0.01[d]
titratable acidity (g citric acid/100 g f.w.)	1.67 ± 0.01[a]	1.76 ± 0.01[a]	1.75 ± 0.05[a]	2.32 ± 0.12[b]
soluble solids (% at 20 °C)	9.2 ± 0.14[a]	9.8 ± 0.06[b]	10.5 ± 0.05[c]	10.0 ± 0.10[b]
moisture content (g/100 g fw)	84.77 ± 0.11[a]	85.31 ± 0.63[a]	83.67 ± 1.53[b]	82.02 ± 3.01[c]
total solids (g/100 g fw)	15.23 ± 0.02[a]	14.69 ± 0.11[a]	16.33 ± 0.30[b]	17.98 ± 0.66[c]
proteins (μg/100 g fw)	82.24 ± 10.46[a]	97.38 ± 3.58[ab]	111.76 ± 9.08[b]	97.60 ± 0.18[ab]
PPO activity (ΔOD/min/g fw)	1.19 ± 0.006[a]	0.83 ± 0.006[b]	0.64 ± 0.01[b]	1.21 ± 0.05[a]

[a] Different letters in the same row indicate significant differences ($p \leq 0.05$); fw fresh weight; values are the mean (±SD) of three determinations.

Table 2. Influence of Extraction Buffer Composition on Raspberry PPO Activity

buffer composition	PPO activity[a] (ΔOD/min/g fw)
0.05 M sodium phosphate (pH 7.0) + 4% (w/v) insoluble PVP	0.125 ± 0.01
0.05 M sodium phosphate (pH 7.0) + 4% (w/v) insoluble PVP + 0.5% (w/v) Triton X-100	0.690 ± 0.02
0.05 M sodium phosphate (pH 7.0) + 4% (w/v) insoluble PVP + 0.1% (w/v) NaCl	0.110 ± 0.03
0.05 M sodium phosphate (pH 7.0) + 4% (w/v) insoluble PVP + 0.5% (w/v) Triton X-100 + 0.1% (w/v) NaCl	0.585 ± 0.02
0.1 M sodium phosphate (pH 7.0) + 4% (w/v) insoluble PVP + 0.5% (w/v) Triton X-100	0.532 ± 0.001
0.2 M sodium phosphate (pH 7.0) + 4% (w/v) insoluble PVP + 0.5% (w/v) Triton X-100	0.425 ± 0.002

[a] Activity values are average of three independent determinations ± standard deviation; fw fresh weight.

temperature with a Crison pH meter. After determination of pH, the solution was titrated with 0.1 N NaOH up to pH 8.1. The results were expressed as citric acid percentage (g citric acid per 100 g fw) (AOAC, 1984).

Soluble Solids. Soluble solids were measured with an Atago digital refractometer dbx-30 at 20 °C. Results were reported as Brix degrees.

Total Solids and Moisture Content. The AOAC (1984) vacuum oven method was modified, using a microwave oven operating at 200 W for 25−30 min, as described in Cano et al. (1990).

Enzyme Extraction. Enzymatic extracts were prepared so that PPO activity was determined at the highest level (Table 2). In all assays, 20 g of pulverized liquid nitrogen frozen pulp was homogenized and mixed with 25 mL of extraction buffer for 2 h at 4 °C in the dark. The homogenates were centrifuged in a Sorvall model RC-5B refrigerated superspeed centrifuge for 30 min at 18000*g* and 4 °C until assayed for PPO activity.

PPO Activity. The PPO activity was determined at 25 °C by measuring the initial rate of increase in absorbance at 420 nm. Unless otherwise stated, activity was assayed in 3 mL of reaction mixture, consisting of 2.7 mL of 0.1 M cathecol in 0.2 M sodium phosphate buffer (pH 5.5) plus 0.3 mL of prepared enzyme, with a Perkin-Elmer spectrophotometer model Lambda 15. Molarity of the reaction buffer was selected as indicated in Table 3. The enzyme activity was determined by measuring the slope of the reaction line at zero time (initial rate). The enzyme activity unit was defined as the change in absorbance/min/mg protein extracted (specific activity) or the change in absorbance/min/g tissue.

Protein Determination. Protein concentrations of the extracts were measured by the Bradford (1976) method, measuring optical density (OD) at 595 nm, with bovine albumin as a standard.

Table 3. Effect of Reaction Mixture Buffer Molarity on Raspberry PPO Activity

buffer composition	PPO activity[a] (ΔOD/min/g fw)
0.2 M sodium phosphate (pH 5.5)[b]	0.690 ± 0.02
0.1 M sodium phosphate (pH 5.5)	0.535 ± 0.03
0.05 M sodium phosphate (pH 5.5)	0.592 ± 0.01

[a] Values are average of three independent determinations ± standard deviation; fw fresh weight. [b] The pH 5.5 was selected by a previous pH study of optimal conditions for spectrophotometric PPO assay.

Substrate Specificity. The substrates used for the specificity study are listed in Table 4. All compounds were prepared in 0.2 M sodium phosphate buffer (pH 5.5).

Effect of pH. A study was made of the effect of pH on the catechol oxidation by papaya PPO. Enzyme activity was determined in 0.2 M sodium phosphate buffer at different pH values, ranging from 4.5 to 9.0.

Effect of Substrate Concentration. Solutions of catechol and catechin varying in concentration from 20 mM to 0.8 M for catechol and from 2 to 10mM for catechin were employed to study the effect of substrate concentration, in Heritage and Ceva raspberry extracts. In a cuvette, 0.3 mL of enzyme solution was mixed with 2.7 mL of catechol or catechin at different concentrations in 0.2 M sodium phosphate buffer at pH 5.5. Michaelis constants (K_m) and maximum velocities (V_{max}) of PPO were calculated from a plot of 1/activity versus 1/substrate concentration by the method of Lineweaver and Burk (1934).

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on a Miniprotean II dual slab cell unit (Bio-Rad). Runs were performed at constant current intensity (35 mA per plate), with cooling to ~4 °C for 30 min. Polyacrylamide gels (10%) were prepared according to Laemmli (1970) without SDS (native conditions). After running, gels were incubated at 10 g/L in a 0.8 M catechol solution in 0.2 M sodium phosphate buffer (pH 5.5) for 15 min. Also, duplicate gels were washed several times with 40% ethanol for an additional 5 min after standard staining with catechol.

Data Analysis. Values are the average of three independent determinations. These results were analyzed for variance (ANOVA) and statistical significance by *t* test with Statgraphics and/or InStat software packages.

RESULTS AND DISCUSSION

Selections of Conditions for Enzyme Assay. Several buffer compositions were employed to select the most suitable to extract PPO from raspberry tissue (Table 2). The increase in molar concentration of sodium phosphate buffer decreases the extraction of PPO activity nearly to 40% when insoluble PVPP and Triton X-100 were also employed. An increase of more than 5-fold in PPO activity was observed using 0.5% (w/v) Triton X-100 and 4% (w/v) insoluble PVPP. The increase of ionic strength by addition of sodium chloride did not increase enzyme activity (Table 2). Therefore, a 0.05 M

Table 4. Effect of Substrate on Raspberry Soluble PPO Activity[a]

	raspberry cultivar			
	Heritage		Ceva	
substrate[b] (0.0075 M)	PPO activity (ΔOD/min/g fw)	PPO activity (ΔOD/min/mg protein)	PPO activity (ΔOD/min/g fw)	PPO activity (ΔOD/min mg protein)
catechin	1.48 ± 0.02	0.075 ± 0.009	1.21 ± 0.08	0.070 ± 0.003
protocatechin	ND	ND	ND	ND
catechol (0.1 M)	0.83 ± 0.006	0.04 ± 0.01	0.64 ± 0.01	0.034 ± 0.006
4-methylcatechol	0.18 ± 0.00	0.083 ± 0.008	ND	ND
caffeic acid	ND	ND	ND	ND
chlorogenic acid	ND	ND	ND	ND
L-dopa	ND	ND	ND	ND
p-coumaric acid	1.48 ± 0.00	0.074 ± 0.008	0.34 ± 0.01	0.019 ± 0.004

[a] Values are average of three independent determinations ± standard deviation; fw fresh weigth; ND not detectable. [b] PPO activity using catechol as substrate was detectable at higher concentration (0.1 M).

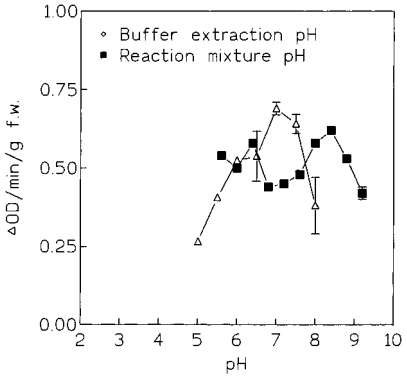

Figure 1. Effect of buffer extraction and reaction mixture pH of spectrophotometric assay on the enzymatic activity of raspberry PPO.

sodium phosphate buffer containing 4% (w/v) insoluble PVPP and 0.5% (w/v) Triton X-100 was employed for all enzyme assays. The use of PVPP has been reported to produce good results in PPO extraction from different plant tissues, such as banana (Galeazzi et al., 1981), strawberry (Wesche and Montgomery, 1990), and papaya (Cano et al., 1996).

Tanning reactions during enzyme extraction can cause partial inactivation of the enzymes. To avoid these reactions, reducing agents are often added during extraction, but must be removed before assay. Alternatively, phenolic substrates must be removed prior to the assay with insoluble PVPP (Mayer and Harel, 1979). In the present work, the use of insoluble PVPP alone was not enough to cleavage all phenolics, and consequently, extracted PPO activity was very low. However, the use of a detergent (Triton X-100), together with insoluble PVPP improved the extraction combining polar and nonpolar binding capacities.

pH Optima for Extraction and Activity. The pH stability was measured by extracting the enzyme in buffers ranging from pH 5.0 to 8.0 (Figure 1). Maximum activity was extracted at pH 7.0 with catechol as substrate. The same pH optimum for extraction was obtained when catechin was used as a substrate. No extractions were attemped at pH lower than 5.0.

pH optima for PPO activity were similar for catechol or catechin as substrates. Two maxima activities, pH 5.5 and 8.0, were observed using catechol reaction (Figure 1) and could relate to the two PPO isoenzyme forms separated by PAGE electrophoresis. However, in this work all enzyme assays were conducted at pH 5.5 due to the solubilization of enzyme extract aliquots in the reaction mixture. The pH of raspberry tissue ranged from 3.65 to 2.65 depending on the fruit cultivar (Table 1). At this pH range, raspberry PPO was not effectively active, but a decrease in acidity of the tissue due to postharvest fruit metabolism could induce browning problems in the fresh fruits. An increase of ionic strength from 0.1 to 0.2 M of sodium phosphate buffer produced a nearly 25% increase in PPO enzyme activity (Table 3).

Substrate Specificity. Heritage raspberry PPO activity was more active with catechin and *p*-coumaric acid using a concentration of 0.0075 M, followed for 4-methylcatechol (Table 4). However, Ceva raspberry activity showed a highest specificity for catechin followed for *p*-coumaric acid, and no activity was observed using 4-methylcatechol as substrate. Raspberry PPO also showed activity using catechol as substrate, but its concentration must be higher (0.1 M), to obtain good reproducibility and measure of the enzyme activity. Catechol was used for all raspberry PPO assays because it is less expensive. Raspberry PPO contains catecholase and cresolase activity, the latter at lower magnitude in Ceva extracts. Sherma and Ali (1980) reported that the active site of the other possible PPO activity, cresolase activity, was more labile than that of catecholase. However, in the present work the cresolase activity was not easily inactivated during the extraction process.

No activity was detected toward protocatechin, caffeic acid, chlorogenic acid, and L-dopa, in any raspberry extracted tissues (Table 4). These compounds are naturally occurring phenolic compounds in raspberry tissues and results toward catechin and *p*-coumaric acid as substrate indicated that raspberries have a great potential for enzymatic browning during postharvest handling or during processing and storage of raspberry-based products.

Effect of Substrate Concentration. The effects of catechol concentrations ranging from 50 mM to 0.8 M and catechin concentrations ranging from 2 to 10 mM on PPO activity of heritage and Ceva raspberries were investigated (Figures 2−5). The K_m and V_{max} values for the PPO were determined from Lineweaver−Burk plots (Figures 3 and 4) for Heritage and Ceva raspberries, respectively, using catechol as substrate. Figures 4 and 6 showed similar plots using catechin. The K_m values for catechol were 663.9 mM for Heritage extracts and 84.2 mM for Ceva raspberries. If catechin was used for enzymatic assays, the K_m values were 475.96 M for Heritage and 699.79 M for Ceva raspberries. The V_{max} values were 4.47 and 1.28 ΔOD/min/g fw for Heritage and Ceva, respectively, using catechol for PPO reaction. Moreover, V_{max} values were 14.135 and 3.919 ΔOD/

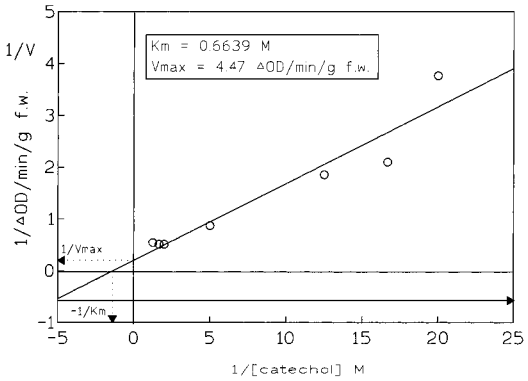

Figure 2. Effect of substrate concentration (catechol) on Heritage raspberry PPO activity (Lineweaver−Burk plot).

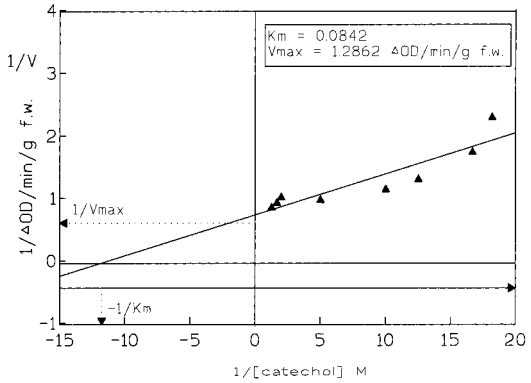

Figure 3. Effect of substrate concentration (catechol) on Ceva raspberry PPO activity (Lineweaver−Burk plot).

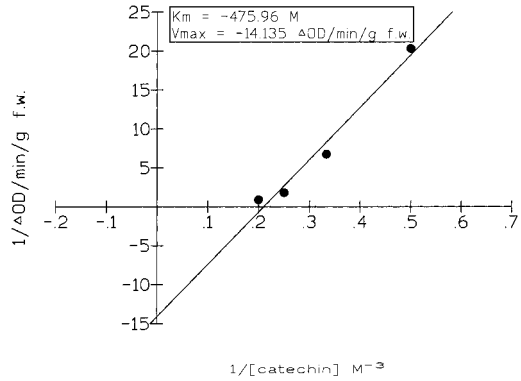

Figure 4. Effect of substrate concentration (catechin) on Heritage raspberryPPO activity.

min/g fw for these two raspberry cultivars in the same order, using catechin as substrate.

PPO Activity on Raspberry Cultivars. Autumm Bliss and Rubi fruits did not show any significant difference in terms of PPO activity (Table 1) ($p \leq 0.05$), 1.19 and 1.21 (ΔOD/min/g fw), respectively. These values are followed by Heritage PPO activity, 0.83 (ΔOD/min/g fw) and Ceva one 0.64 (ΔOD/min/g fw). Raspberry PPO from all four cultivars studied was not active toward chlorogenic acid, but it was very active using catechin and *p*-coumaric as substrates (Table 3). This indicates that raspberries have a great potential for enzymatic browning when they are processed.

Electrophoresis. Polyacrylamide gel electrophoresis on 10% gels under native conditions resolved crude PPO extracts in one band (Figure 6). To determine the active isoenzyme of PPO, solutions of different substrates were assayed according to the procedures described for PPO

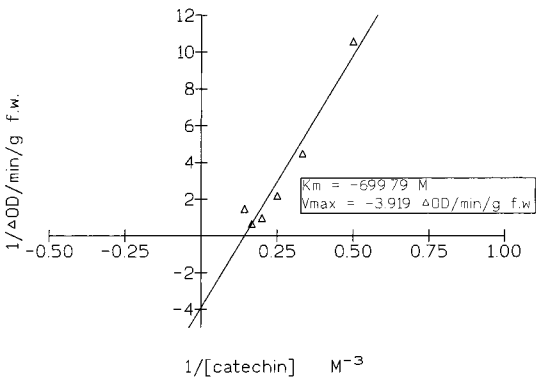

Figure 5. Effect of substrate concentration (catechin) on Ceva raspberry PPO activity.

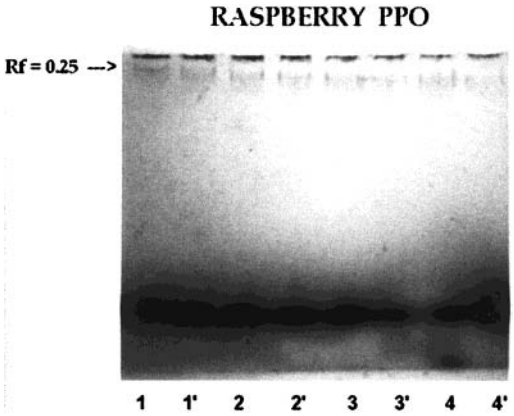

Figure 6. Electrophoretic pattern of soluble PPO activity from rapsberry: 1,1′ cv. Heritage; 2, 2′ cv. Autumm bliss; 3,3′ cv. Ceva; 4,4′ cv. Rubi. Staining gel was with 0.8 M catechol.

substrate affinity. Solutions for affinity were 0.7 and 0.8 M catechol; 0.01 and 0.02 M catechin in 0.2 M sodium phosphate buffer (pH 5.5). Staining the gels for 10 min was enough to visualize the only PPO band. However, the use of 0.8 M catechol solution rendered the better PPO isoenzyme visualization. Duplicate gels were also washed several times with ethanol to produce a partial gel dehydration in order to bring out other possible PPO bands, following a protocol reported by Galeazzi et al. (1981) and employed in other studies with papaya PPO (Cano et al., 1996). However, in the present work the use of ethanol did not improve the staining of raspberry PPO. Figure 6 shows duplicates of soluble PPO pattern of the four raspberry cultivars. All raspberries have the same PPO isoenzyme band with a mobility of $R_f = 0.25$, which was stable after staining with 40% ethanol washings.

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