Polyphenol Oxidase from Apple (*Malus domestica* Borkh. cv Bramley's Seedling): Purification Strategies and Characterization

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ABSTRACT: Polyphenol oxidase (PPO) was isolated from Bramley's Seedling apples with 75.7-fold purification and 26.5% recovery by ammonium sulfate precipitation, phenyl sepharose chromatography, ion exchange chromatography, and hydroxyapatite chromatography. Molecular weight was estimated to be about 45 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE). Optimum PPO activity was at pH 6.5 and greater than 50% activity was retained during storage for 72 h at pH 5.5 to 6.5. Optimum temperature for activity was 30 °C and the enzyme had residual activity of greater than 50% during storage for 72 h at 20 °C to 30 °C and for 24 h at 40 °C to 50 °C. Of the substrates tested, activity was greatest with 4-methylcatechol followed by catechol, pyrogallol, and (-)-epicatechin. The most effective inhibitors tested were sodium metabisulfite and ascorbic acid.

Keywords: polyphenol oxidase, apple, characterization, purification, diphenolase activity

Introduction

Polyphenol oxidases (PPOs) are copper-containing enzymes that catalyze the hydroxylation of monophenols to *o*-diphenols (cresolase or monophenol monooxygenase activity EC 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (catecholase or diphenolase activity EC 1.10.3.1). These *o*-quinones then undergo subsequent reactions to form dark-colored compounds. PPOs are responsible for the enzymatic browning reaction that occurs in damaged fruits or vegetables (Eskin 1990; Lee 1991; Mayer and Harel 1991). These enzymes have the potential to crosslink macromolecules containing suitable phenols and this may be of value in the texture engineering of foods.

PPO has been isolated and characterized in various fruits including apples (Harel and others 1964; Janovitz-Klapp and others 1989; Trejo-Gonzalez and Soto-Valdez 1991; Murata and others 1992; Zhou and others 1993; Espín and others 1995; Oktay and others 1995), peaches (Flurkey and Jen 1980), kiwifruit (Park and Luh 1985), pears (Rivas and Whitaker 1973), and plums (Siddiq and others 1992). During extraction of PPO, it is essential to minimize phenol oxidation, which can result in activity losses due to reaction of quinones with the enzyme (Smith and Montgomery 1985; Rocha and Morais 2001). Methods to remove phenolics from the extraction medium include addition of phenol-binding agents such as polyvinylpyrrolidone (PVP) and AG2-X8 resin (Smith and Montgomery 1985; Wissemann and Montgomery 1985; Wesche-Ebeling and Montgomery 1990; Zhou and others 1993; Gauillard and Richard-Forget 1997). Protease inhibitors are used in the extraction of PPO to minimize generation of artifactual multiple forms of enzyme (Flurkey and Jen 1980).

PPO from Bramley's Seedling leaf has been partially purified (Ridgway and Tucker 1999), but there is an absence of information on purification and characterization of PPO from Bramley's Seedling apple fruit. In this work, characterization of PPO from Bramley's Seedling apples was studied in terms of pH optimum and stability, heat optimum and stability, substrate specificities, and degrees of inhibition by general PPO inhibitors, to help predict the behavior of the apple enzyme.

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Materials and Methods

Apples

Bramley's Seedling apples harvested at commercial maturity, in September, at The Apple Farm, County Tipperary, Ireland, and stored at 4 °C, were used. Data presented relate to the purification of PPO from apples within 1 mo of their harvest.

Measurement of PPO activity

PPO activity was assayed using the procedure of Oktay and others (1995). The reference cuvette contained 3.0 mL substrate (50 m*M* catechol substrate in 0.2 *M* sodium phosphate buffer pH 7.0) and the sample cuvette contained 2.9 mL substrate and 0.1 mL enzyme solution. The reaction was carried out at 25 °C and increase in absorbance at 420 nm was measured using a dual-beam recording spectrophotometer. Change in absorbance was recorded every 3 s for 1 min and enzyme activity was measured from the linear portion of the curve (Wong and others 1971). One unit of PPO activity was defined as the change in absorbance of 0.001 per min per mL of enzyme. Activity measurements were carried out in triplicate.

Extraction procedure

Unless stated otherwise, all extraction procedures were carried out at 4 °C to reduce enzyme activity and all chemicals were from Sigma Aldrich, Dublin, Ireland. Apple slices (375 g, after peeling and core removal) were blended for 1 min with 600 mL 0.2 *M* sodium phosphate buffer, pH 7.0, containing 45 g AG2-X8 resin (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) and 45 g polyvinylpolypyrrolidone (PVPP). Protease inhibitors, aprotinin 0.33 mL/100 mL and

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2 m*M* phenylmethylsulfonylfluoride (PMSF) were added immediately. The solution was stirred for 1 h and then centrifuged at 15000 × *g* for 30 min and the supernatant filtered through Whatman nr 4 filter paper. Aprotinin (0.33 mL/100 mL) and 0.1 m*M* aminoethylbenzenesulfonylfluoride (AEBSF) were added to the filtrate.

Purification procedure

The apple filtrate was treated with solid ammonium sulfate, 30% to 85% saturation. Precipitate was collected by centrifugation at 15000 × *g* for 30 min and redissolved in buffer (50 m*M* sodium phosphate buffer, pH 7.0, containing 1.2 *M* ammonium sulfate and 1.2 *M* potassium chloride) and dialyzed overnight against 3 changes (3 × 1 L) of the same buffer. The enzyme extract was centrifuged at 34500 × *g* for 30 min and the supernatant collected. AEBSF (0.1 m*M*) and aprotinin (0.33 mL/100 mL) were added to the supernatant.

Chromatography was carried out at room temperature using a Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Chalfont St. Giles, Bucks, U.K.). Dialyzed extract was applied onto phenyl-sepharose 4-fast flow (Amersham Biosciences), packed into a XK 16/20 column (Amersham Biosciences, 7 × 1.6 cm) previously equilibrated with the dialysis buffer (equilibration buffer). The column was washed with 70 mL equilibration buffer to remove unbound proteins. Decreasing equilibration buffer concentration stepwise from 100% to 0% with distilled water eluted the bound proteins. The flow rate was 1.0 mL/min and 2 mL fractions were collected. Absorbance at 280 nm and PPO activity were determined for each fraction. The most active fractions were combined and dialyzed overnight against 3 changes of equilibration buffer (3 × 1 L) at 4 °C. AEBSF (0.1 m*M*) and aprotinin (0.33 mL/100 mL) were added to dialyzed active enzyme fractions.

The sample was then reapplied to the same phenyl-sepharose 4-fast flow column; 40 mL equilibration buffer was then run through the column to remove unbound proteins; bound proteins were eluted by decreasing equilibration buffer concentration from 100% to 0% over 100 mL. Flow rate was 1.0 mL/min and 2-mL fractions were collected. Absorbance at 280 nm and PPO activity were determined for each fraction. The most active fractions were combined and dialyzed overnight against 3 changes (3 × 1.5 L) of 20 m*M* Tris-HCl, pH 7.0, buffer. AEBSF (0.1 m*M*) and aprotinin (0.33 mL/100 mL) were added to the dialyzed active enzyme fractions. The column was regenerated after each run by washing with 4 volumes distilled/deionized water, 4 volumes of 1 *M* NaOH, and 4 volumes distilled/deionized water.

Dialyzed enzyme extract was then applied onto a Mono Q HR 5/5 (bed volume 1 mL) ion exchange column (Amersham Biosciences), which had been equilibrated (changing counter ions) at room temperature by washing with 5 mL low-ionic-strength buffer (20 m*M* Tris-HCl, pH 7.0) followed by 10 mL high-ionic-strength buffer (20 m*M* Tris-HCl, pH 7.0, containing 1.0 *M* potassium chloride) and finally with-low-ionic strength buffer (20 m*M* Tris-HCl pH 7.0) until a stable base line was established. After sample application, the column was washed with 15 mL low-ionic-strength buffer. Flow rate was 0.8 mL/min and 1-mL fractions were collected. Proteins were eluted by gradually increasing to the high-ionic-strength buffer (elution buffer). Four linear gradients were used during the elution process: the 1st was from 0% to 16% elution buffer over a volume of 17 mL; the 2nd was from 16% to 19% over 5 mL; the 3rd was from 19% to 25% over 20 mL; and the 4th was from 25% to 100% over 20 mL. The final wash with 100% elution buffer was applied over 12 mL. Absorbance at 280 nm and PPO activity were determined for each fraction. Three peaks of PPO activity were eluted from the ion exchange column and labeled IE1, IE2, and IE3. Peak IE2 contained the majority of PPO activity and was dialyzed against 3 changes (3 × 1 L) of 1 m*M* sodium phosphate buffer, pH 7.0, at 4 °C. The Mono Q Column was cleaned and regenerated according to the manufacturer's instructions.

AEBSF (0.1 m*M*) + aprotinin (0.33 mL/100 mL) were added to the dialyzed enzyme fractions from the ion exchange chromatography. IE2 was applied to a Macro-Prep ceramic hydroxyapatite (Type 2, particle size 80 μm; Bio-Rad Laboratories) packed onto a XK 16/20 column (Amersham Biosciences) (7 × 1.6 cm). The column had been previously equilibrated at room temperature with 1 m*M* sodium phosphate buffer, pH 7.0. After sample application, the column was washed with 50 mL of 1 m*M* sodium phosphate buffer, pH 7.0. The flow rate was 0.8 mL/min and 2-mL fractions were collected. Applying a linear gradient against 50 m*M* sodium phosphate buffer, pH 7.0, containing 1 *M* potassium chloride over a volume of 40 mL eluted the bound proteins from the column. A final wash with 20 mL elution buffer was applied. Absorbance at 280 nm and PPO activity were determined for each 2-mL fraction. The column was cleaned and regenerated according to the manufacturer's instructions.

Protein determination

Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin as the standard. Bradford reagent (1.5 mL) was added to 0.05 mL of sample. Absorbance at 595 nm was determined using a single-beam spectrophotometer.

Effect of pH

pH activity was examined by adding 0.1 mL enzyme solution to 2.9 mL of 50 m*M* catechol in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, ranging from pH 2.5 to pH 9.0 and PPO activity assayed as described previously. pH stability was determined by incubating 0.2 mL enzyme with 0.4 mL of 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, ranging from pH 2.5 to pH 9.0, for up to 72 h at 20 °C. Residual PPO activity was assayed periodically as described previously.

Substrate specificity

Substrate specificity was determined by using 9 different substrates (catechol, tyrosine, pyrogallol, 4-methylcatechol, (-)-epicatechin, caffeic acid, L-3,4-dihydroxyphenylalanine [DL-dopa], ferulic acid, and phenol). Substrate solutions were prepared in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5, at 3 different concentrations (10 m*M*, 2.5 m*M*, 1 m*M*). PPO activity was assayed as described previously.

Effect of temperature

PPO activity was measured at different temperatures in the range 20 °C to 80 °C as described previously. Thermal stability of the enzyme was determined by incubating the enzyme at various temperatures between 20 °C and 80 °C for up to 7 h. Residual PPO activity was assayed periodically as described previously.

Effect of inhibitors

PPO activity was measured in the presence of 12 different inhibitors (ascorbic acid, sodium chloride, sodium metabisulfite, L-cysteine, citric acid, ethylenediaminetetraacetic acid (EDTA) disodium salt, N-acetyl-L-cysteine, succinic acid, borax, calcium chloride, benzoic acid, and potassium sorbate) at 3 concentrations (10 m*M*, 1 m*M*, 0.1 m*M*) in 50 m*M* catechol (pH 7). PPO activity was assayed as described previously, and results were reported as percent catechol inhibition.

Electrophoresis

Enzyme purity was determined at various stages of the purification process using sodium dodecyl sulfate-polyacrylamide gel elec-

Table 1—Purification of polyphenol oxidase (PPO) from Bramley's Seedling apples[a]

Purification step	Volume (mL)	Activity (units/mL)	Total activity (units)	Protein content (mg/mL)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
Crude	720	665	478800	0.221	3009	1	100
$(NH_4)_2SO_4$ precipitation	55	7457	410135	0.831	8977	3	85.7
Phenyl sepharose 1	58	5877	340837	0.238	24691	8.2	71.2
Phenyl sepharose 2	69	4132	287139	0.106	39161	13	60
Ion exchange							
IE peak 1	4	355	1329	0.103	3459	1.1	0.3
IE peak 2	19	7562	143669	0.409	185104	61.5	30
IE peak 3	12	760	9114	0.158	4822	1.6	1.9
Hydroxyapatite of IE peak 2	23	5521	126972	0.024	227650	75.7	26.5

[a]PPO activity was assayed using 50 m*M* catechol in 50 m*M* sodium phosphate buffer, pH 7.0. Values are typical recovery results.

trophoresis (SDS PAGE) (Laemmli 1970) and a Bio Rad Gel System (Amersham Biosciences). A 12% separating gel and a 4% stacking gel were used. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 or Silver Stain Kit. Nondenaturing, native PAGE was performed without the use of SDS. Native gels were stained for PPO activity with 50 m*M* catechol in 0.2 *M* sodium phosphate buffer, pH 7.0.

Results and Discussion

Table 1 presents a summary of the results for PPO extraction and purification from Bramley's Seedling apples. Specific activity for the crude apple PPO extract was 3009 units/mg. Precipitation with ammonium sulfate (30% to 85%) resulted in a 3-fold increase in specific activity to an average of 8977 units/mg with a recovery of 85.7% of the crude extract.

Three peaks containing PPO activity were separated by phenyl sepharose chromatography. One large peak was eluted at 80% to 60% equilibration buffer and 2 smaller peaks at 100% and 20% to 0% equilibration buffer (Figure 1).

The major peak corresponded to an 8.2-fold increase in specific activity over the crude extract and a column yield of 83% of total activity units applied. Fractions from the major peak containing highest PPO activity were combined, dialyzed, and reapplied to the phenyl sepharose column. Two peaks containing PPO activity were eluted, a large peak at 80% to 60% equilibration buffer and a small peak at 20% to 0% equilibration buffer (Figure 2). The major peak corresponded to a 13-fold increase in specific activity over the crude extract and a column yield of 84% of total activity units applied to the column.

Hydrophobic interaction chromatography using phenyl sepharose has been utilized in the purification of PPO from various fruits including apples (Janovitz-Klapp and others 1989; Zhou and others 1993), peaches (Flurkey and Jen 1980), grapes (Wissemann and Lee 1980), pears (Wissemann and Montgomery 1985), strawberries (Wesche-Ebeling and Montgomery 1990), pineapple (Das and others 1997), and apricot (Chevalier and others 1999). Purification factors, elution profiles, and enzyme recovery have varied between studies. Reported purification factors were 161-fold for PPO from Monroe apple peel (Zhou and others 1993), 120-fold for PPO from Red Delicious cortex (Janovitz-Klapp and others 1989), and 13.8-fold for PPO from apricot (Chevalier and others 1999). Recoveries of 40% for PPO from Monroe apple peel (Zhou and others 1993), 45% for PPO from Red Delicious cortex (Janovitz-Klapp and others 1989) and 80% for PPO from apricot (Chevalier and others 1999) have been reported.

Fractions containing PPO activity were combined, dialyzed, and applied to an ion exchange column (Mono Q HR). Three peaks (IE1, IE2, IE3) containing PPO activity were eluted from the Mono-Q column at 80% to 75%, 75% to 60%, and 20% to 0% equilibration buffer representing a 1.1-fold, 61.5-fold, and 1.6-fold increase in specific activity over the crude extract and yields of 0.5%, 50%, and 3.2% over the previous step, respectively (Figure 3). Chromatography

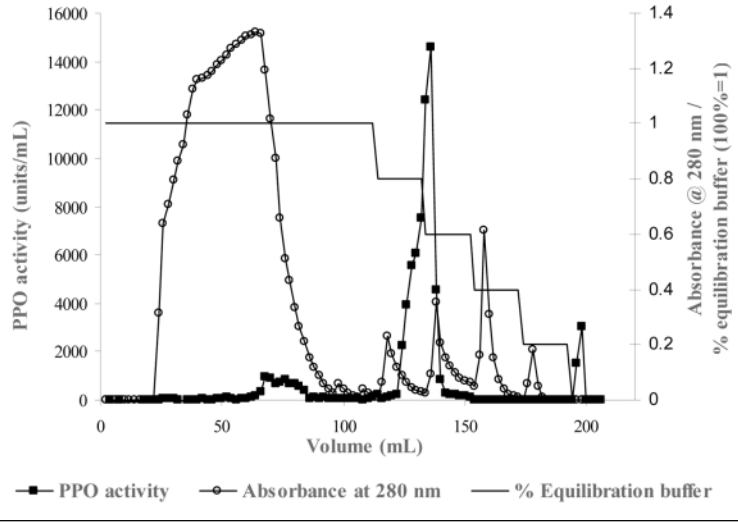

Figure 1—Absorbance at 280 nm and polyphenol oxidase (PPO) activity of fractions collected from Phenyl Sepharose 4 Fast Flow chromatography of PPO from Bramley's Seedling apples. PPO activity was assayed using 50 m*M* catechol in 50 m*M* sodium phosphate buffer, pH 7.0. Data represent typical recovery results.

with different ion exchange media has previously been used in the purification of PPO from various sources including Bartlett pears (Rivas and Whitaker 1973), Fuji apple (Murata and others 1992), apricot (Chevalier and others 1999), Bramley's Seedling leaf (Ridgway and Tucker 1999), kiwi fruit (Park and Luh 1985), and peaches (Flurkey and Jen 1980).

The most active fractions from peak IE2 were combined, dialyzed, and applied to a hydroxyapatite column. One peak containing PPO activity was eluted at 70% to 90% equilibration buffer, which represented a 75.7-fold increase in specific activity over the crude extract and a column yield of 88% of total activity units applied to the column (Figure 4). Hydroxyapatite chromatography has also been used in the purification of PPO from peach (Flurkey and Jen 1980) and pears (Rivas and Whitaker 1973). Overall yield of activity was 26.5% and specific activity was increased 75.7-fold to an average of 227650 units/mg protein.

Characterization of Bramley's Seedling PPO

Effects of pH on activity and stability. Optimum pH for activity of PPO from Bramley's Seedling apple was found to be 6.5 (Figure 5). Optimal values of pH 6 to 7 with catechol as substrate have also been reported (Table 2) for other apple cultivars (Oktay and others 1995; Rocha and others 1998; Weemaes and others 1998) and for various fruits including peach (Wong and others 1971), kiwifruit (Park and Luh 1985), plum (Siddiq and others 1992), avocado, grape and pear (Weemaes and others 1998), and pineapple (Das and others 1997). Activity for PPO from Bramley's Seedling apples dropped sharply above pH 7; similar results have been reported for apple, pear, grape, avocado, and plum (Weemaes and others 1998), pear (Halim and Montgomery 1978), and pineapple (Das and others 1997).

Relative activity below pH 4.5 was less than 10%. Similarly, very low activity below pH 4.5 has also been reported for Stanley plum (Siddiq and others 1992) and medlar fruit (Dincer and others 2002), and below pH 4.0 for apple, grape, plum, pear, and avocado PPO (Weemaes and others 1998). However, this inactivity at low pH is at variance with several studies of apple PPO where the pH optima were reported to be between pH 4.5 and 5.5 (Rivas and Whitaker 1973; Trejo-Gonzalez and Soto-Valdez 1991; Richard-Forget and others 1992; Zhou and others 1993; Nicolas and others 1994).

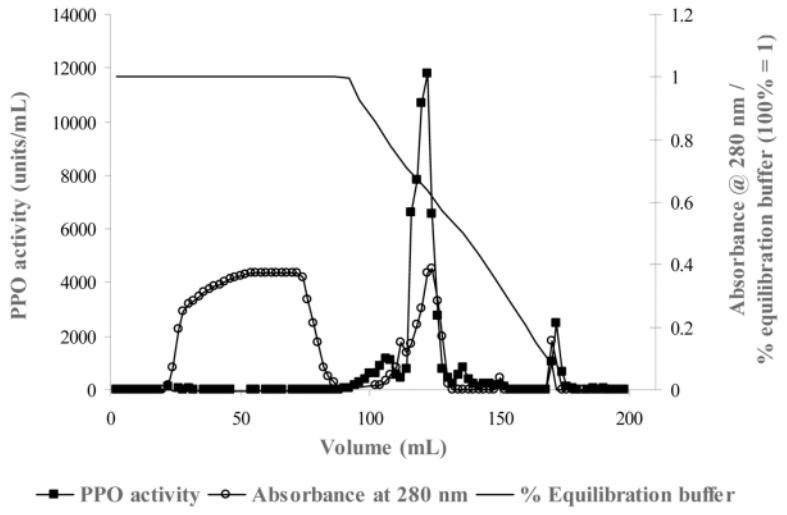

Figure 2—Absorbance at 280 nm and polyphenol oxidase (PPO) activity of fractions collected from s Phenyl Sepharose 4 Fast Flow chromatography step. PPO activity was assayed using 50 m*M* catechol in 50 m*M* sodium phosphate buffer, pH 7.0. Data represent typical recovery results.

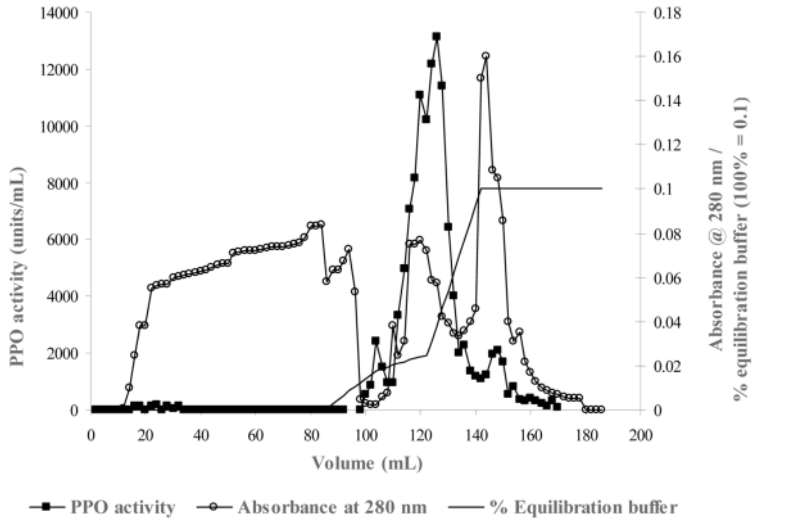

Figure 3—Absorbance at 280 nm and polyphenol oxidase (PPO) activity of fractions collected from Mono Q HR column. PPO activity was assayed using 50 m*M* catechol in 50 m*M* sodium phosphate buffer, pH 7.0. Data represent typical recovery results.

Bramley's Seedling apple PPO was found to be most stable at pH 5.5 to 6.5, maintaining greater than 50% activity during storage for 72 h, and most unstable at pH 2.5 to 3.5, retaining less than 10% of original activity after 2 h (Figure 6). More than 50% activity was retained after 48 h at pH 5.0, 7.0, and 7.5, and after 24 h at pH 4.5, 8.0, and 8.5.

The enzyme retained greater than 50% of its original activity when stored at pH 9.0 for 4 h (Figure 6). Instability at low pH has also been reported for pear below pH 3.5 (Rivas and Whitaker 1973), medlar fruit below pH 4.0 (Dincer and others 2002), and plum below pH 4.5 (Siddiq and others 1992).

Effects of temperature on activity and stability

Optimum temperature for apple PPO activity was found to be 30 °C (Figure 7). Similar temperature optima of between 25 °C and 35 °C have been found using catechol as substrate (Table 2) for other apple cultivars (Trejo-Gonzalez and Soto-Valdez 1991; Zhou and others 1993), Concord grape (Cash and others 1976), and medlar fruit (Dincer and others 2002).

Bramley's Seedling apple PPO was found to have residual stability of greater than 50% after incubation at 20 °C to 30 °C for 72 h, 40 °C to 50 °C for 24 h, 60 °C for 1 h, and 70 °C for 15 min (Figure 8).

Heating at 80 °C for 15 min inactivated the enzyme. Rapid decline in activity at temperatures greater than 50 °C has been reported for PPO from different fruit sources (Wissemann and Lee 1980; Siddiq and others 1992; Zhou and others 1993; Dincer and others 2002).

Substrate specificity. Data on substrate specificity for PPO from Bramley's Seedling apples are outlined in Table 3. Highest PPO activity was found using 4-methylcatechol, followed by catechol, pyrogallol, (-)-epicatechin, caffeic acid, and DL-dopa. Little or no activity was detected toward the monophenolic compounds ferulic acid, L-tyrosine, and phenol.

Tyrosine has previously been reported to be a poor substrate for apple PPO (Zhou and others 1993; Rocha and others 1998; Rocha and Morais 2001). Studies have also shown 4-methylcatechol to be a better substrate than catechol (Table 2) for PPO from apple (Harel and others 1964; Murata and others 1992; Zhou and others 1993; Rocha and others 1998; Rocha and Morais 2001), plum (Siddiq and others 1992), peach (Flurkey and Jen 1980), medlar fruit (Dincer

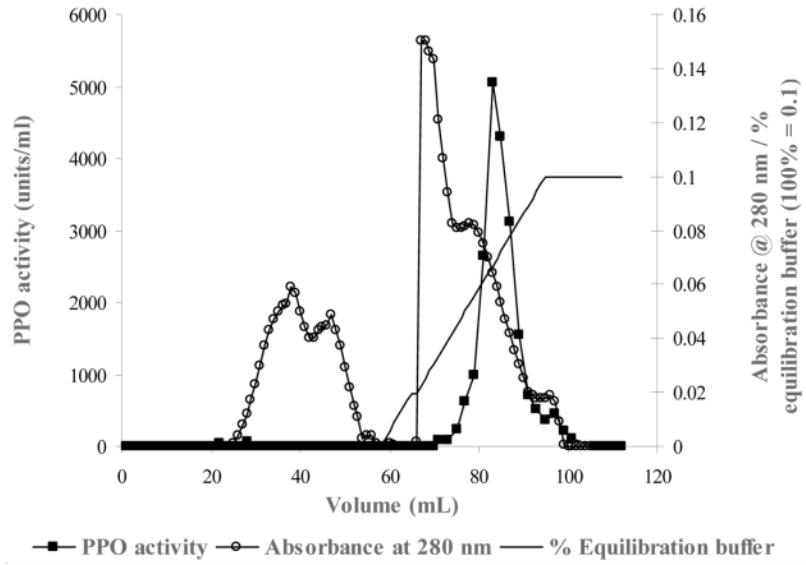

Figure 4—Absorbance at 280 nm and polyphenol oxidase (PPO) activity of fractions collected from hydroxyapatite chromatography. PPO activity was assayed using 50 m*M* catechol in 50 m*M* sodium phosphate buffer, pH 7.0. Data represent typical recovery results.

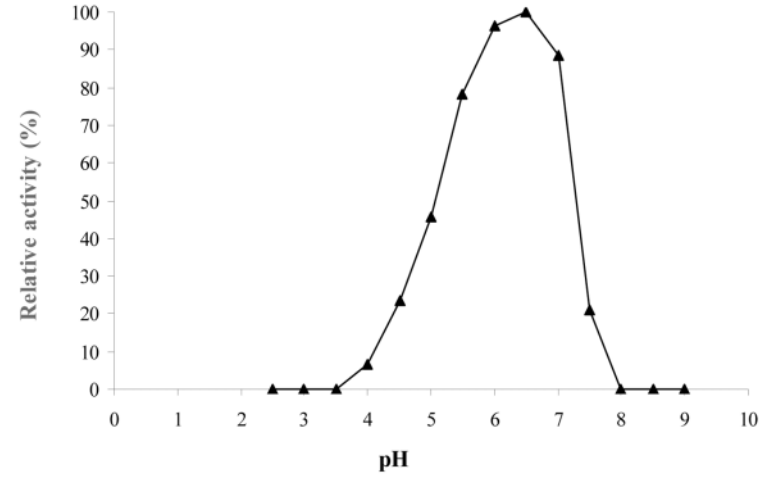

Figure 5—Effects of pH on activity of polyphenol oxidase (PPO) from Bramley's Seedling apples in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer (pH 2.5 to 9.0). Activities are expressed as % relative activity compared with activity determined with 50 m*M* catechol in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5. Values are means for duplicate analyses of 2 replicates. Standard deviations of all data were less than 10%.

and others 2002), and strawberry (Wesche-Ebeling and Montgomery 1990). As was found in the present study, PPO from Bramley's Seedling leaf had greater affinity for *o*-diphenolics than monophenolics (Ridgway and Tucker 1999). Phenolic compounds found in apples include chlorogenic acid, (-)-epicatechin, caffeic acid, ρ-coumaric acid, catechin, catechol, 3,4-dihydroxyphenylalanine (dopa), procyanidin B2, (+)-catechin, and phloridzin. Of these, chlorogenic acid is the major polyphenol in mature apples (Vamos-Vigyazo 1981; Eskin 1990; Nicolas and others 1994).

Effect of inhibitors. Inhibition of PPO from Bramley's Seedling apple PPO is shown in Table 4. Of the inhibitors used, sodium metabisulfite was found to be the most effective, followed by ascorbic acid, L-cysteine, N-acetyl-L-cysteine, benzoic acid, and citric acid (Table 4).

Table 2—Characteristics of some polyphenol oxidases from various fruit sources

PPO source	Optimum pH (catechol substrate)	Optimum temperature (°C, catechol substrate)	Substrate specificity	Inhibitor efficiency
Apple cv. Bramley's Seedling (present study)	6.5	30	4-methylcatechol > catechol > pyrogallol > (-)-epicatechin > caffeic acid > DL-dopa. No activity toward L-tyrosine, ρ-coumaric acid.	Sodium metabisulfite > ascorbic acid > L-cysteine > N-acetyl-L-cysteine > benzoic acid > citric acid > succinic acid > EDTA disodium salt > calcium chloride > borax > potassium sorbate > sodium chloride.
Apple cv. Amasya (Oktay and others 1995)	7.0	18	Catechol > 4-methylcatechol > pyrogallol > L-dopa.	L-cysteine > sodium acid > sodium cyanide > β-mercaptoethanol > glutathione > thiourea.
Apple cv. Jonagored (Rocha and Morais 2001)	5.0, 7.5	—	L-dopa > 4-methylcatechol > (+)-catechin >ρ-cresol > dopamine > catechol > L-tyrosine.	Sodium metabisulfite > L-cysteine > ascorbic acid > thiourea > ρ-coumaric acid > benzoic acid.
Plum cv. Stanley (Siddiq and others 1992)	6.0	20	4-methylcatechol > catechol > dopamine > pyrogallol > caffeic acid > DL-dopa > chlorogenic acid > L-tyrosine > hydroquinone > gallic acid > ρ-cresol.	Sodium metabisufite = L-cysteine > ascorbic acid > thiourea > ρ-coumaric acid > benzoic acid.
Strawberry (Wesche Ebeling and Montgomery 1990)	5.5	—	D-catechin > 4-methylcatechol > pyrogallol > cholorgenic acid > caffeic acid > catechol > protocatechuic acid > dopa. No activity toward L-tyrosine, ρ-coumaric acid.	Potassium cyanide > dithiothretol > diethyldithiocarbamate > potassium metabisulfite > benzenesulfinic acid > L-cysteine > sodium chloride.
Monroe Apple Peel (Zhou and others 1993)	5.0	30	4-methylcatechol > chlorogenic acid > catechol > D-catechin > pyrogallol > dopamine > DL-dopa > tyrosine.	Thiourea > L-cysteine > sodium diethyldithiocarbamate > ascorbic acid > potassium metabisulfite > 2-mercapotethanol > sodium cyanide.
Pineapple (Das and others1997)	6-7	—	Catechol > L-dopa > catechin > chlorogenic acid.	Ascorbic acid > L-cysteine > potas sium metabisulfite > cinnamic acid > resorcinol > EDTA.

[a]EDTA = ethylenediaminetetraacetic acid; PPO = polyphenol oxidase.

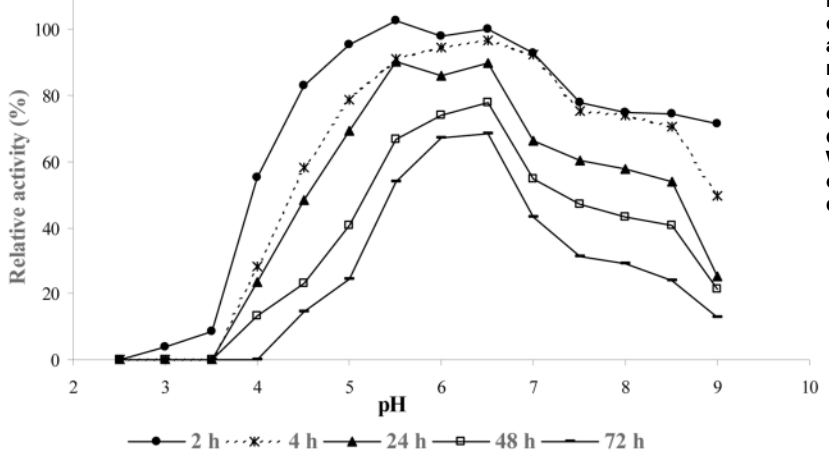

Figure 6—pH stability of polyphenol oxidase (PPO) from Bramley's Seedling apples. Activities are expressed as % relative activity compared with activity determined at pH 6.5 with 50 m*M* catechol in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5. Values are means for duplicate analyses of 2 replicates. Standard deviations of all data were less than 10%.

Table 3—Substrate specificity for polyphenol oxidase (PPO) from Bramley's Seedling apples[a]

Substrate	Concentration (m*M*)	Relative activity (%)
Catechol	10.0	100
	2.5	30
	1.0	9
Pyrogallol	10.0	80
	2.5	40
	1.0	12
4-Methylcatechol	10.0	220
	2.5	120
	1.0	55
(-)-Epicatechin	2.5	71
	1.0	46
Caffeic acid	10.0	50
	2.5	40
	1.0	32
DL-dopa	2.5	28
	1.0	9
L-tyrosine	2.5	0
	1.0	0
Ferulic acid	2.5	0
Phenol	10.0	0

[a]Activities are expressed as % relative activity compared with activity determined with 10 m*M* catechol. Substrates were prepared in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5. Values are means for duplicate analyses of 2 replicates. Standard deviations of all data were less than 10%.

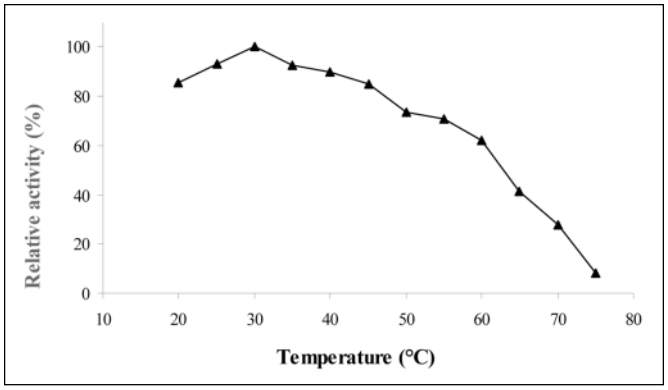

Figure 7—Effects of temperature on activity of polyphenol oxidase (PPO) from Bramley's Seedling apples in buffer. Enzyme was assayed for each temperature after an incubation of 30 min. Activities are expressed as % relative activity compared with activity determined at 30 °C with 50 m*M* catechol in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5. Values are means for duplicate analyses of 2 replicates. Standard deviations of all data were less than 10%.

Sodium chloride was the weakest inhibitor. Sodium metabisulfite, ascorbic acid, and L-cysteine have been shown to be strong inhibitors of PPO (Table 2) from Stanley plum (Siddiq and others 1992), Monroe apple peel (Zhou and others 1993), and medlar fruit (Dincer and others 2002).

Table 4—Effect of inhibitors on polyphenol oxidase (PPO) from Bramley's Seedling apple[a]

Inhibitor	Concentration (m*M*)	Inhibition (%)
Ascorbic acid	10.0	99
	1.0	97
	0.1	79
Sodium chloride	10.0	22
	1.0	12
	0.1	0
Sodium metabisulfite	10.0	100
	1.0	100
	0.1	90
L-cysteine	10.0	100
	1.0	100
	0.1	45
Citric acid	10.0	98
	1.0	81
	0.1	30
EDTA disodium salt	10.0	52
	1.0	36
	0.1	6
N-acetyl-L-cysteine	10.0	99
	1.0	99
	0.1	52
Succinic acid	10.0	89
	1.0	67
	0.1	19
Borax	10.0	41
	1.0	24
	0.1	55
Calcium chloride	10.0	37
	1.0	29
	0.1	52
Benzoic acid	10.0	96
	1.0	73
	0.1	60
Potassium sorbate	10.0	35
	1.0	19
	0.1	13

[a]Activities are expressed as % relative activity compared with activity determined with 10 m*M* catechol. Substrates were prepared in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5. Values are means for duplicate analyses of 2 replicates. Standard deviations of all data were less than 10%. EDTA = ethylenediaminetetraacetic acid.

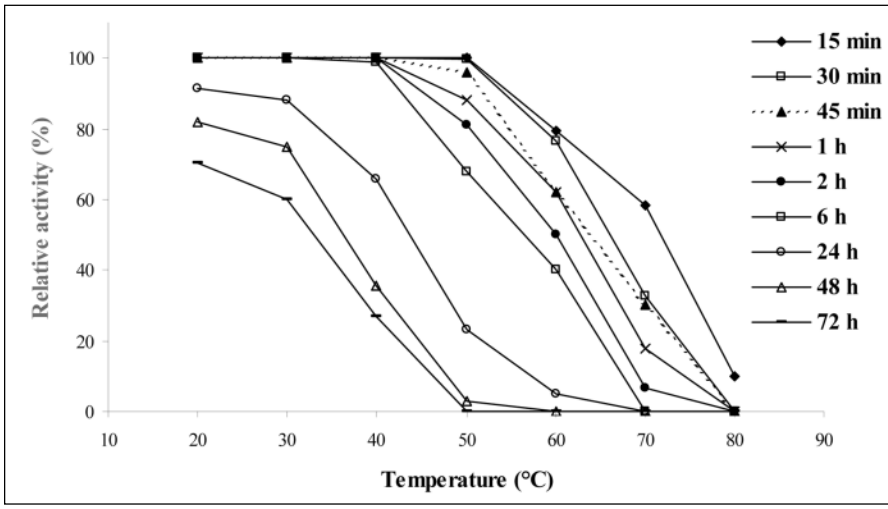

Figure 8—Temperature stability for polyphenol oxidase (PPO) from Bramley's Seedling apples. Activities are expressed as % relative activity compared with initial activity determined with 50 m*M* catechol in 0.1 *M* citric acid–0.2 *M* disodium phosphate buffer, pH 6.5. Values are means for duplicate analyses of 2 replicates. Standard deviations of all data were less than 10%.

Electrophoresis. The molecular weight and purity of apple PPO extracts were examined by SDS-PAGE. The molecular weight of PPO from Bramley's Seedling apples was estimated to be 45 kDa on SDS-PAGE with another protein band observed at around a few thousand kDa (Figure 9). The enzyme produced a single band on native PAGE following staining with catechol (Figure 9).

These results indicate that the purified enzyme has a molecular weight of about 45 kDa. Similar molecular weights of 46 kDa for Red Delicious cortex apple PPO (Janovitz-Klapp and others 1989), 42 kDa for pineapple fruit (Das and others 1997), and 38-39 kDa for grape (Sanchez-Ferrer and others 1989) have been reported. Other values reported for apple PPO include 65 kDa (Murata and others 1992), 26 kDa (Goodenough and others 1983), and several forms of 30-40, 60-70, and 120-130 kDa (Harel and others 1965).

Conclusions

This work describes a successful method for the separation and purification of PPO from Bramley's Seedling apples. It can be concluded that this PPO is a catecholase, active toward diphenols, and has greatest substrate specificity toward 4-methylcatechol among the substrates tested. Consistent with previous results for PPOs from other plant sources, optimum pH was 6.5 and optimum temperature 30 °C. The PPO inhibitors, sodium metabsulfite, ascorbic acid, and L-cysteine were particularly effective.

Acknowledgments

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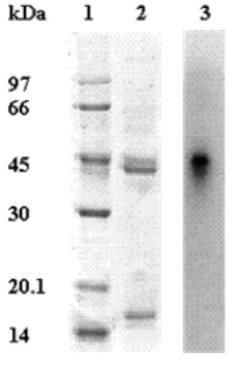

Figure 9—Purification of polyphenol oxidase (PPO) from Bramley's Seedling apples: Lane 1 = molecular weight markers; lane 2 = Apple PPO sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE); lane 3 = Apple PPO nondenaturing PAGE substrate staining with catechol.