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POLYPHENOLOXIDASE FROM APPLE, PARTIAL PURIFICATION AND SOME PROPERTIES

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Key Word Index—*Malus domestica*; Rosaceae; apple fruit; polyphenoloxidase; purification.

Abstract—The optimum conditions for polyphenoloxidase (PPO) extraction from apple fruit were obtained with a buffer solution above pH 7 containing 15 mM ascorbic acid and 0.5% of Triton X100. In 12 cultivars grown in France, the PPO activity ranged from 3.1 to 0.62 in the cortex and from 3 to 0.3 mkat/kg in the peel. For both parts, the cultivar Red Delicious exhibited the highest activity, whereas the lowest activity was in Elstar. In the Red Delicious apples, the PPO activity steadily declined during the eight weeks around the commercial date of harvest, the total decrease being close to 30%. PPO from Red Delicious cortex has been purified 120-fold, with a total yield close to 40%, by ammonium sulphate precipitation and hydrophobic chromatography on Phenyl Sepharose CL4B. The pIs, obtained by isoelectrofocusing were at pH 4.5 and 4.8 for two major bands and near neutral for a minor one. The M_r determined by gel filtration was unique (*ca* 46 000). The optimum pH of activity was between 4.5 and 5 for 4-methylcatechol, chlorogenic acid and (+) catechin. The K_m values were almost independent of pH on the acid side of the pH optimum and were in the range of 5 mM for the three substrates studied. At pH 4 which is close to the pH of apple vacuoles, chlorogenic acid is a better substrate for apple PPO than (+) catechin.

INTRODUCTION

Enzymatic browning of raw fruits and vegetables is mainly due to the oxidation of natural phenolic compounds into quinones which, in turn, are polymerized to brown, red or black pigments [1]. The main enzyme involved in this reaction is polyphenoloxidase (EC 1.14.18.1:PPO) which has been the subject of many reviews [2–5]. PPO purified from numerous fruits and vegetables [6] exhibits multiple forms. However, this multiplicity may be the consequence of endogenous phenolics oxidation during the extraction process [7] resulting in aggregated forms of PPO.

After the first works of Weurman and Swain [8, 9], Nakabayashi [10] and Siegelman [11], many studies have been devoted to apple PPO with contradictory results. Thus, according to Harel *et al.* [12] and Macheix [13], total PPO activity drops in the later stages of apple fruit development whereas the reverse was found by Zocca and Ryugo [14]. Considerable variations were found in the relative activity towards different phenols from one preparation to another [15–18]. Harel *et al.* [19] and Walker and Hulme [20] have partially purified PPO from apple peel using DEAE cellulose chromatography, whereas Stelzig *et al.* [21] used calcium phosphate gel adsorption. After electrophoresis the number of isoforms varied from one [22], two [20, 23] to three [19, 24] which according to Harel and Mayer [25] could be due to various degrees of subunits aggregation of the same enzyme. The optimum pH of activity ranged from 4.2 to 7.3 [15, 21–23, 26, 27].

In order to clarify some of these discrepancies and obtain further information on the different parameters related to the degree of browning of apple cultivars, the need for a stable and purified PPO extract is obvious. It was the purpose of this work to obtain such a preparation and investigate some of its properties.

RESULTS AND DISCUSSION

Preliminary experiments have been performed in order to set the optimum conditions for PPO extraction. Thus, in presence of Triton X100 and ascorbic acid, the pH must be raised above pH 7 since at pH lower than 6, the extracted activity was less than 15% of the maximum activity obtained at pH 7.2. The extracted activity was very sensitive to Triton, as it was almost absent when the detergent was omitted, it increased rapidly up to 0.5% Triton and then reached a plateau. An optimum of ascorbic acid was observed corresponding to 15 mM in the extraction medium.

Twelve apple cultivars were analysed for PPO activity both in cortex and in peel (Table 1). Among the cultivars, the PPO activity level in the cortex part ranged from 3.1 to 0.62 and from 3 to 0.3 mkat/kg in the peel part. In both cases, the Red Delicious cultivar had the highest PPO activity whereas the Elstar cultivar had the lowest. In recent studies, Coseteng and Lee [28] and Klein [29] also found that PPO activity was highest in the Red Delicious cultivars. With the exception of the Mutsu cultivar, the PPO activity was always either equivalent or lower in the peel than in the cortex. Some authors claimed that the activity was mainly concentrated in the peel [21, 27], whereas others found the reverse [29, 30]. Such differences may be due not only to the different cultivars studied, but also to the extraction method used.

As the Red Delicious cultivar was the highest in PPO activity, it was chosen for the maturity study and as the

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Table 1. PPO activity in peel and cortex of 12 apple cultivars (Results are expressed in mkat/kg of lyophilized tissue)

Cultivar	Peel	Cortex	Cultivar	Peel	Cortex
Red Delicious	3.04	3.10	Canada	1.46	2.73
MacIntosh	1.40	2.48	Granny Smith	1.30	2.26
Fuji	1.74	2.20	Mutsu	2.17	1.67
Gala	0.90	1.49	Jonagold	1.30	1.33
Florina	1.27	1.24	Charden	0.93	1.21
Golden Delicious	0.99	0.93	Elstar	0.31	0.62

enzyme source for the purification. The change in PPO activity in the four weeks preceding and the three following the commercial harvest date, is shown in Fig. 1. The general trend was a decrease in PPO activity of *ca* 30% during this period. This result is in agreement with those already published [12, 13, 28] although, in an equivalent period, i.e. one month before and after the commercial harvest date, the Granny Smith cultivar exhibited almost no change in the PPO activity (data not shown).

Typical results obtained during the purification steps are shown in Table 2. After extraction, the ammonium sulphate treatments and the dialysis allowed a four-fold purification with a yield of 45%. The profile obtained in the last step (hydrophobic chromatography) is illustrated in Fig. 2. All PPO activity was eluted in a single peak with less than 4% of the applied proteins. The yield of chromatography was always better than 80%, leading to an overall purification factor of 120 for the four pooled fractions. Furthermore, for the most active fraction, which accounted for 12% of the original crude extract, activity was 150-fold purified. the purity of the enzyme preparation was checked by isoelectrofocusing (IEF) in polyacrylamide gels (Fig. 3). Specific stain revealed two major bands in the 4.5–5 region and one minor faint band whose pI value was near neutral, whereas protein staining indicated that our PPO preparation was still contaminated by inactive proteins. The IEF in liquid medium performed in a 3.5–10 pH gradient confirmed the presence of a broad peak of PPO activity whose maximum was at pH 4.8 accompanied by a shoulder at pH 4.5. It also showed the presence of *ca* 30% of inactive proteins. A minor peak, less than 5% of the recovered activity, was also apparent at pH 6.7 (Fig. 4). The values for pI of apple PPO have not been published before. However, they are consistent with the conditions used for DEAE cellulose chromatography, namely pH 7.2 [19] and 5 [20], in the published PPO purification procedures from apple peel.

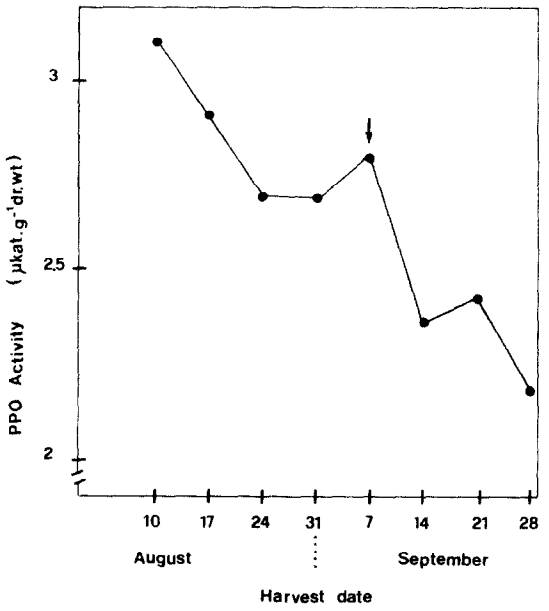

Fig. 1 Changes in PPO activity during maturation of Red Delicious cultivar (arrow indicates the commercial harvest).

Despite the heterogeneity of the apple PPO system, it always eluted in a single peak during gel filtration using a calibrated column of AcA 54. The obtained M_r was 46 000, value which is rather different from those previously found, namely 30–40, 60–70 and 120–130 000 [25] and 26 000 [22] by gel filtration on Sephadex G100. Maintained at 4°, the purified PPO was relatively stable, losing less than 10% of the original activity in 15 days, whereas held at 30°, half of the original activity was lost in 4 hr at pH 4.5. Similar results have been obtained by Goodenough *et al.* [22].

At each pH, the initial velocity was determined as a function of concentrations for 4-methylcatechol, chlorogenic acid and (+)-catechin. The effect of pH on K_m and V_m is given in Table 3 for the three substrates studied. The relative maximum value for V_m was obtained in the same pH region namely 4.5 to 5 for all the substrates. K_m and V_m values were slightly higher for 4-methylcatechol than for chlorogenic acid between pH 4.5 and 5. However at these pHs, the (+)-catechin oxidation rate was *ca* half of that observed for the two other substrates. Similar results were obtained by Stelzig *et al.* [21] at pH 4.2 using the substrates at a 20 mM concentration. Moreover, many authors used pH values between 4.5 and 5 for their assays of apple PPO activity [15, 19–22, 26–28]. When the pH was lower than 5, the K_m values were almost constant whatever the substrate. Therefore, hydrogen ion can be considered as a linear non-competitive inhibitor for apple

Table 2. Results obtained during purification of apple PPO from 20 g of lyophilized cortex (cultivar Red Delicious)

Step	Total volume (ml)	Total protein (mg)	Total activity (μkat)	Specific activity (μkat/mg)	Yield %	Purification factor
Crude extract	230	43	53.8	1.25	—	—
Ammonium sulphate 30%	240	40.3	54	1.34	100	1.1
Ammonium sulphate 80%	41	4.6	24.2	5.25	45	4.2
Phenyl Pooled fractions	28	0.14	20.4	150	38	120
Sepharose Most active	7	0.035	6.6	188	12	150

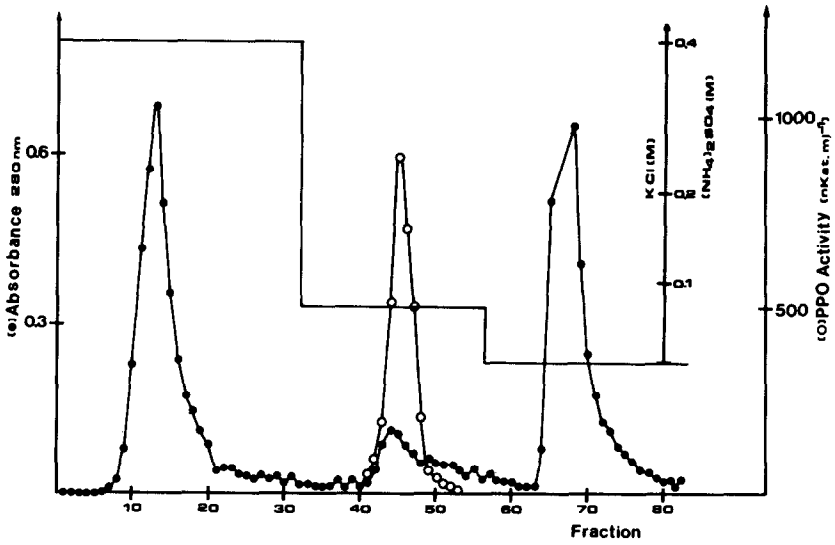

Fig. 2. Hydrophobic chromatography of apple PPO on Phenyl Sepharose CL4B.

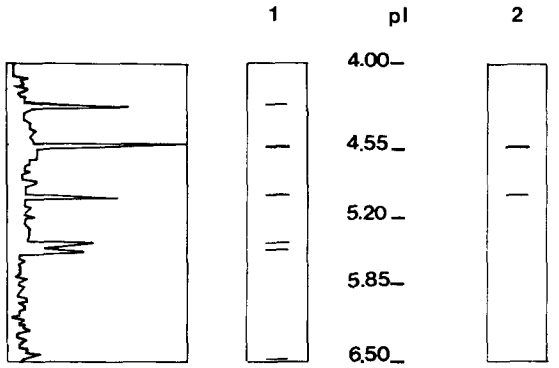

Fig. 3. Isoelectrofocusing in polyacrylamide gels (pH 4–6.5) of the purified fraction of apple PPO after hydrophobic chromatography of Phenyl Sepharose CL4B. Lane 1: silver nitrate staining with its corresponding densitogram on the left, lane 2: specific staining of PPO activity. The migration distances of the markers obtained by densitometry are given between lane 1 and lane 2.

PPO on the acid side of the optimum pH. The K_m independence of pH was also shown for purified PPO from pears using chlorogenic acid as substrate [31]. A pK value of 3.65 was calculated for the prototropic group of apple PPO involved in the oxidation of 4-methylcatechol and not its binding to enzyme since pH does not affect K_m value. On the alkaline side of the optimum pH, where both K_m and V_m increased indicating a mixed-type inhibition, a pK value of 5.5 was obtained. In another way, the oxidation rate was more sensitive to pH variation below 5 for (+)-catechin than for the other two substrates. Thus, at pH 4 which is close to the pH of apple vacuoles, the velocity was decreased by 40% for (+)-catechin whereas it was still more than 80% of the maximum for 4-methylcatechol and chlorogenic acid. Therefore, at these acid pHs and after bruising, chlorogenic acid is a better substrate than (+)-catechin for apple PPO from a kinetic point of view. However, from a browning point of view, further information is needed to decide which compound is the most important.

EXPERIMENTAL

Materials. For the maturation study, samples of 18 apples, var. Red Delicious, were picked from 6 trees on August 10, 17, 24, 31 and September 7, 14, 21 and 28, 1987 in the experimental orchard of the Centre Technique Interprofessionnel des Fruits et Légumes at Balandran near Avignon. For the cultivar study, 100 apple fruits from 12 cultivars, grown either at Balandran or at the Station d'Amélioration des Espèces Fruitières de l'INRA at Angers, were picked at commercial maturity. Immediately after picking, 18 fruits of each cultivar were individually peeled. The peel was lyophilized whereas the unseeded cortex was crushed in liquid N_2 before freeze-drying. Both parts of each fruit were individually stored at −20° until use. For the purification study and after the Red Delicious cultivar was selected, freeze-dried cortex was prepared from 30 kg of fruits picked at commercial maturity from Angers using the above procedure. Ultrogel AcA 54 was from IBF Biotechnics. PhenylSepharose CL4B and the different Phastgels were from Pharmacia. Chlorogenic acid and (+)-catechin were from Extrasynthèse and all other chemical were reagent grade quality supplied by Sigma.

Extraction procedure. 2 g of lyophilized material were suspended in 25 ml of cold McIlvaine's buffer at pH 7.2 containing 0.5% Triton X100 and 15 mM ascorbic acid, for 30 sec using an Ultra-Turrax blender and held for 15 min. The homogenate was centrifuged (40 000 *g*, 40 min) at 4° and the supernatant was used as the crude extract.

Purification procedure. All steps were carried out at 4° with the exception of the hydrophobic chromatography which was performed at room temp. Starting from 10 times the above quantities, a 30–80% satn in $(NH_4)_2SO_4$ was prepared from the crude extract containing 1% of $CaCl_2$. The resulting pellet was resuspended in 40 ml of 0.05 M Na-Pi buffer at pH 6.5, 0.4 M in $(NH_4)_2SO_4$ and KCl and dialysed overnight against the same buffer. After centrifugation (40 000 *g*, 30 min), the supernatant was applied onto a Phenyl Sepharose CL4B column (8 × 2.5 cm, 40 ml bed vol.) equilibrated with the same buffer at a flow rate of

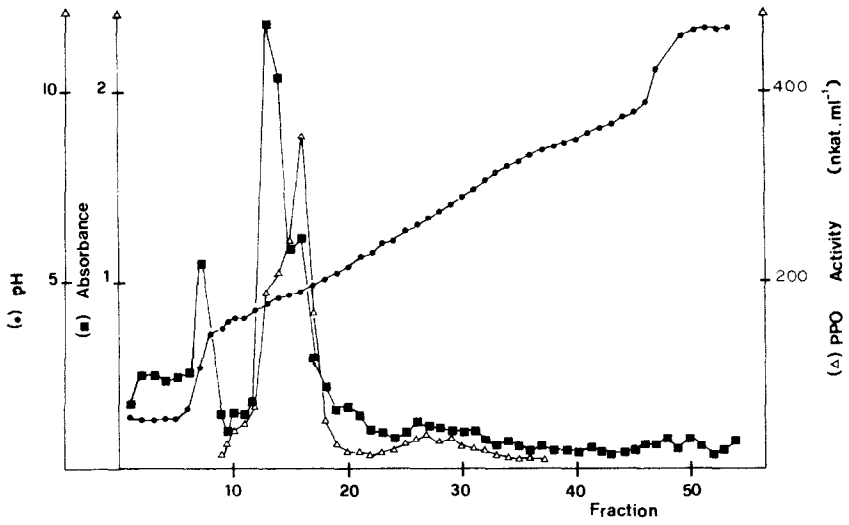

Fig. 4. Isoelectrofocusing experiment in liquid medium of purified apple PPO. IEF was performed at 4° for three days in a pH gradient of 3.5–10. On each fraction of 1.5 ml collected, pH was measured at 30°, protein was determined at 280 nm and activity was assayed by polarography.

Table 3. K_m and V_m parameters for apple PPO activity as a function of pH for 4-methylcatechol, chlorogenic acid and (+) catechin

	4-Methylcatechol		Chlorogenic acid		(+)-Catechin	
pH	K_m (mM)	V_m (%)	K_m (mM)	V_m (%)	K_m (mM)	V_m (%)
3.5	5.2	47				
4	5.3	80	4.1	82	6.1	35
4.5	5.2	99	4.1	93	6.3	50
5	5.2	100	4.25	90	6.2	55
5.2	6.5	82				
5.5	8.1	70	4.9	70	6.6	48
5.7	13.3	58				
6.3	20.5	20				

V_m values are expressed as % of the maximum value obtained at pH 5 for 4-methylcatechol which was equal to 25 nkat.

100 ml/hr. After elution of unbound proteins by the equilibration buffer, the PPO activity was eluted using the same buffer, but 0.1 M in $(NH_4)_2SO_4$ and KCl. Proteins still bound to the gel were removed by H_2O and 50% ethylene glycol in H_2O. The A at 280 nm and the PPO activity were determined on each 7 ml fraction.

IEF experiments and M_r *determination.* IEF in polyacrylamide gels was performed with the Phastsystem (Pharmacia) using Phastgels pH 3–9 and 4–6.5. The migration and $AgNO_3$ staining conditions were those described in the Phastsystem booklet. Gels stained for PPO activity were immersed in the substrate soln containing *p*-phenylenediamine (0.05%). The pH gradient profile across the gel was determined using the broad pI calibration kit from Pharmacia. Ultroscan XL densitometer from LKB was used for the determination of the migration distances of marker and unknown proteins. IEF in liquid medium was carried out at 4° with a 110 ml column (LKB Type 8101) in the pH range 3.5–10 as already described [32]. A column packed with Ultrogel AcA 54 (90 × 0.8 cm, 180 ml bed vol.) was calibrated with the gel filtration calibration kit of low M_r proteins from Pharmacia. 1.5 ml of purified PPO (or standard proteins) was loaded onto the column equilibrated with 0.1 M Na-Pi buffer at pH 6.5 containing 0.5 M NaCl and eluted at a flow rate of 7.5 ml/hr. The A at 280 nm and PPO activity were determined on each 4 ml fraction.

PPO and protein assays. The substrate was 20 mM in 4-methylcatechol in McIlvaine's buffer at pH 4.5. PPO activity was routinely assayed by polarography at 30° using air-satd substrate soln. Activity was expressed as nmol of O_2 consumed per sec (nkat). Due to the Clark electrode used, the oxygen uptake remained proportional to the enzyme amount until the limit of 25 nkat. For the study of the pH effect on K_m and V_m, concns were varied from 20 to 2 mM for 4-methylcatechol and from 10 to 1 mM for chlorogenic acid and (+)-catechin. For each pH and each substrate, assays were performed in duplicate. K_m and V_m values were determined using a non-linear regression data analysis program for IBM PC developed in ref. [33]. The protein content was determined by the method of ref [34] using bovine serum albumin as standard.

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