Characterization of polyphenoloxidase (PPO) extracted from 'Jonagored' apple

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Abstract

Polyphenoloxidase (PPO) was extracted from apple (cv. Jonagored) with addition of 2% PVP and 0.25% Triton X100 to the extraction buffer containing phenolic adsorbents. Experiments were performed to evaluate the affinity and specificity towards several substrates. 'Jonagored' apple PPO was found to have higher specificity (lower K_m) towards L-dopa, 4-methylcatechol and (+) catechin than other phenols tested, but the highest activity level was obtained with p-cresol. The ratio V_{max}/K_m indicates that p-cresol followed by L-dopa and 4-methylcatechol are the best substrates for 'Jonagored' apple PPO. The enzyme activity showed two pH optima, at 5.0 and 7.5, at room temperature, with the main peak at pH 7.5 and the secondary one at pH 5.0 when catechol was the substrate.

1. Introduction

Polyphenoloxidase (PPO) activity has been extensively reported, by several authors, to be the main factor involved in apple browning (Nicolas, Richard-Forget, Goupy, Amiot & Aubert, 1994). Varietal difference in enzyme activity and susceptibility to browning is a well-known phenomenon. 'Jonagored' is a mutant of 'Jonagold' which is a controlled cross of 'Jonathan' with 'Golden Delicious' (Trillot, Masseron & Trovel, 1993). Although PPO has been investigated for many apple varieties, 'Jonagored' has not yet been studied. The characterization of the specific enzyme is necessary for a more effective means of controlling the enzymatic browning, or if a better understanding of the browning mechanism under specific storage conditions is required. Such information is important for the post-harvest handling of 'Jonagored' and, in particular, for minimal processing operations.

Two main problems are found in the optimization of the extraction conditions of PPO: the difficulty in obtaining full solubilisation of the membrane-bound PPO, and avoiding phenolic oxidation during and after extraction. The strength of PPO binding to membranes is variable. Therefore, in most cases, full extraction of the enzyme requires the use of a detergent such as Triton X100 (Galeazzi & Sgarbieri, 1981; Zhou, Smith & Lee, 1993). The second problem arises from the simultaneous presence of quinones and their endogenous phenolic precursors in crude extracts of the enzyme. It is essential to minimize the formation of quinones, which may react with the enzyme, resulting in activity losses. Several methods have been described to prevent the reaction of phenols with PPO, including the use of phenol-binding agents such as soluble and insoluble polyvinyl pyrrolidone and polyvinilpolypyrrolidone (PVP and PVPP) (Galeazzi & Sgarbieri, 1981). Several investigators successfully used PVP in plant enzyme extraction due to its ability to bind to the phenols and, therefore in preventing phenol–protein interaction (Galeazzi & Sgarbieri, 1981).

The contribution of a given substrate to enzymatic discoloration depends on its concentration and on the nature of the other substrates present in the tissue. In apples, (+) catechin, (−) epicatechin and chlorogenic acid have been identified as substrates of PPO. The catechins were found to be oxidized more rapidly than chlorogenic acid, epicatechin contributing more to browning than the other two compounds. However, since the concentration of chlorogenic acid in apples is several times that of the catechins, its role in browning may be more important (Vámos-Vigyázó, 1981).

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Polyphenoloxidase isoenzymes isolated from higher plants are able to oxidize a wide range of monophenols and o-diphenols with highly variable kinetic parameters, such as the maximum velocity (V_{max}) and the Michaelis–Menten constant (K_m), for different phenols. The K_m is generally interpreted as a measure of affinity of the enzyme for the substrate. The affinity of plant PPO for the phenolic substrates is generally low (high K_m values, 2–6 mM) (Nicolas et al., 1994). In some fruits, the best substrate of PPO is a compound not occurring as a phenolic constituent (Vámos-Vigyázó, 1981). Simple phenols such as catechol are commonly used in phenolase assays in spite of not always being found in association with the enzyme. However, the presence of a potential substrate does not ensure its role as an actual substrate in phenolase action leading to enzymatic browning (Vámos-Vigyázó, 1981).

As enzymatic browning of apple is pH dependent, this is important for the control of discoloration. Although PPO activity is negligible at the natural pH value of the fruit, it may be sufficient to cause browning (Vámos-Vigyázó, 1981). It is generally agreed that pH undoubtedly affects the K_m value (Nicolas et al., 1994). Most previous studies have indicated that, although the optimum pH for the activity of PPO extracted from apple mitochondria is around 7, the PPO of the whole apple tissue has a maximum activity at pH between 4.5 and 5.5 (Janovitz-Klapp, Richard-Forget & Nicolas, 1989; Trejo-Gonzalez & Soto-Valdez, 1991; Richard-Forget, Rouet-Mayer, Goupy, Philippon & Nicolas, 1992; Zhou et al., 1993). Moreover, the enzyme seems to be relatively tolerant of acidic pH.

With the objective of subsequently controlling enzymatic browning mediated by PPO in this specific apple variety 'Jonagored', work was carried out to characterize the enzyme involved. Experiments were performed in order to optimize the extraction conditions of PPO (best ratio of extraction buffer/mass of fruit tissue, best PVP concentration, best pH of extraction) and to evaluate the affinity and specificity of the enzyme toward several substrates.

2. Materials and methods

2.1. Plant material

Apples (cv. Jonagored) were grown at Estação Regional de Fruticultura e Vitivinicultura – Quinta de Sergude, Felgueiras, Portugal. The harvest was on 25 September. The fruit were stored in air at 4°C for 1–3 months until used in the experiments.

2.2. Characterization of apple maturity

The apples were initially characterised in terms of titratable acidity, pH and soluble solids content.

Aliquots (20–30 g) of apple juice from 10 crushed apple cubes were diluted with 250 ml of recently boiled water. Samples (25 ml) of the prepared juice were titrated to pH 8.1 with 0.1 N NaOH. This potentiometric titration was performed with a pH-combined electrode Ingold U402-57/120 and a Crison Micro pH 2002 (Crison Instruments, S.A., Barcelona, Spain) potentiometer. The results were expressed as percentage of malic acid [(ml NaOH$\times$0.1 N/weight of sample titrated in grams)$\times$0.067$\times$100].

The pH was measured in the juice of the crushed apple, using a pH meter Crison, model Micro pH 2002 (Crison Instruments, S.A., Barcelona, Spain) which had been previously standardized to pH 2 and pH 7, and a xerolyte electrode Ingold Lot 406-MG-DXK-57/25, which had been previously standardized to pH 4 and pH 7.

The soluble solid contents of the non-diluted juice from crushed apple cubes was determined at 20°C with a hand-held refractometer, model Atago-ATC1. Data was expressed in degrees Brix.

2.3. Characterization of PPO in 'Jonagored' apple

In order to determine if any portion of the apple flesh was most favorable for PPO extraction, a preliminary experiment was performed, in which a slice of fresh apple was immersed in 0.07 M catechol solution and the surface colour changes subjectively evaluated according to the procedure described by Bolin, Stafford, King & Huxsoll (1977) and Galeazzi & Sgarbieri (1981). The entire surface of the slice darkened in a similar way, which meant that there is no portion of the apple flesh preferentially favorable for PPO extraction.

2.4. Extraction of PPO

Some modifications were introduced to the extraction procedure described by Galeazzi & Sgarbieri (1981). According to results obtained in a preliminary study with 'Starking' apple (Rocha, Galeazzi, Cano & Morais, 1998), it was assumed that tissue storage condition before PPO extraction influenced the efficiency of extraction. The large amount of samples to be analyzed precluded the possibility of assaying for the PPO activity using fresh-cut samples. Therefore, since extracts from 'Starking' cut apple stored at −80°C retained approximately 90% of their initial activity, all the extraction and characterization studies with 'Jonagored' were performed with frozen samples (−80°C), after thawing, in order to assure the same conditions before experiments. Several extraction conditions were tested for frozen apple in order to select those that resulted in higher PPO activities.

The best concentration of insoluble polyvinilpirrolidone (PVP) in the range from 1–4% was determined

first, and then, using the selected PVP concentration, the influence of the ratio of buffer/apple (ml/g) was tested in order to select the ratio that resulted in the highest PPO activities.

The apple samples were homogenized with 0.2 M pH 6.5 sodium phosphate buffer (extraction buffer) with a T 25 basic Ultra-Turrax (IKA Labortechnik) in an external ice bath for 3 min in 1 min intervals. The homogenates were centrifuged at 4°C for 30 min at $16,500 \times g$ (Sorvall RC-5C, Instruments Dupont, refrigerated superspeed centrifuge). The supernatant was filtered through cheesecloth and its volume determined for enzymatic activity assay. Three replicates were assayed for each determination.

2.5. Assay for PPO activity

Enzymatic activity was assayed by measuring the rate of increase in absorbance at a given wavelength (variable for different substrates) at 25°C in a double beam model UV-1601 UV/VIS spectrophotometer at 420 nm. The reaction mixture contained 3.0 ml of substrate solution and different quantities of enzyme (fixed for each substrate). The reference cuvette contained only the substrate solution. The straight-line section of the activity curve as a function of time was used to determine the enzyme activity (Units/g of fruit/min). A unit of enzyme activity was defined as the change of 0.001 in the absorbance value under the conditions of the assay (Galeazzi & Sgarbieri, 1981). When a lag phase occurred, the reaction rate was measured after the lag phase. All the determinations were performed in triplicate.

2.6. Influence of pH of extraction

For the determination of the optimum pH for apple PPO extraction, the optimized extraction conditions (best ratio buffer/fruit) and 0.2 M sodium phosphate buffer (Satjawatcharaphong, Rymal, Dozier & Smith, 1983; Trejo-Gonzalez & Soto-Valdez, 1991) were used. The pH was varied between 4.5 and 8.0. The assay of the PPO activity was performed by adding 200 μl of the enzyme extract to 2.8 ml of 0.16 M catechol (prepared in 0.05 M sodium phosphate buffer, pH 6.5). The enzyme activity was monitored at 420 nm using the spectrophotometric procedure described above. Three replicates were performed for each determination.

2.7. Determination of kinetic parameters

The phenols, used without further purification, were obtained from the following sources: catechol, *p*-cresol, chlorogenic acid, (+) catechin, dopamine, dihydroxy phenylalanine (L-dopa), 4-methylcatechol from Sigma Chemical Co., and tyrosine from Merck. The Michaelis–Menten constant (K_m) was determined with variable substrate concentrations in the standard reaction mixture, at the wavelength of maximum absorption for the correspondent chromophore. The assay cuvettes (3 ml) contained the substrate solution prepared in 0.05 M sodium phosphate buffer at pH 6.5 and a given quantity of the enzyme, which was different for different substrates. For each substrate, data were plotted as 1/activity vs 1/substrate concentration. K_m and V_{max} were determined as the reciprocal absolute values of the intercepts on the *x*- and *y*-axis, respectively, of the linear regression curve (Lineweaver & Burk, 1934).

The enzyme extracts used were obtained from frozen apple samples stored at −80°C for about three months.

2.8. Protein content

The protein content was determined in all preparations used for PPO assay by the calorimetric method described by Bradford (1976). The values were obtained by graphic interpolation on a calibration standard curve with bovine serum albumin (BSA) at 595 nm.

2.9. Polyacrylamide gel electrophoresis

Apple PPO was separated into multiple forms by a modification of the polyacrylamide gel electrophoresis (PAGE) procedure described by Davis (1964). A Bio-Rad Miniprotean II dual slab cell was used for the electrophoresis. Bisacrylamide gels at 7.5% were used according to Laemmli (1970), but under native conditions (i.e., without sodium dodecyl sulfate, SDS). Gels were incubated over 1 h in a solution of 40% methanol (v/v), 10% acetic acid (v/v) and 0.1% Coomassie Brilliant Blue R-250 (w/v) using a shaker (Rotator Heidolph). The gels were then discolored in a solution identical to the one described above but without the colorant to reveal the protein bands. The gels were dried under vacuum in a Bio-Rad Gel Dryer, model 583 for 2 h at 80°C and the relative mobilities (RM) were calculated.

3. Results and discussion

Some chemical properties that enable the characterization of the degree of ripeness of the apples used in this experiment are in Table 1. The apples were at the ripe maturity stage at which they are normally used for consumption.

3.1. Extraction of PPO

Polyvinilpyrrolidone (PVP) and Triton X100 concentrations used for the extraction were 2% PVP and 0.25% Triton X100. Under these conditions, extracts remained clear on standing overnight at 4°C. Lower

Table 1
Some chemical characteristics of 'Jonagored' apple

Chemical characteristics	Mean	S.D.[a]
Soluble solids (° Brix)	12.4[b]	1.2
Titratable acidity (mg malic acid/g fresh product)	0.42	0.14
pH	3.4	0.32

[a] SD are standard errors.
[b] Data are means of three replicates.

Table 2
Influence of the ratio of buffer to apple tissue on PPO activity of 'Jonagored' apple tissue stored at −80°C[a]

Ratio (ml/g)	Insoluble PVP (%)	PPO Activity (U/g fruit/min)	Protein (µg/g fruit)
0.75	2	1499d[b]	0.13
1.0	–	1154e	0.10
1.4	–	1393d	0.12
1.7	–	3344a	0.22
2.5	–	2750b	0.21

[a] Substrate was 0.16 M catechol dissolved in 0.05 M sodium phosphate buffer pH 6.5 ($\lambda = 420$ nm).
[b] Mean separation in columns by Duncan's multiple range test, $P = 0.05$.

levels of PVP resulted in browning of the extract, due to rapid substrate auto-oxidation. The best ratio of buffer/apple sample (ml/g) was found to be 1.7. Increasing the ratio beyond 1.7 did not result in higher PPO activities, probably due to an increased difficulty in homogenization and enzyme solubilisation (Table 2).

3.2. Influence of pH of extraction

The pH activity profile for the oxidation of catechol by 'Jonagored' PPO is shown in Fig. 1. Two pH optima were observed for PPO activity at room temperature (20°C): one at pH 5.0 and the other at pH 7.5. Most assays of PPO activity that have been reported have been carried out between 20°C and 35°C. A significant thermal inactivation may occur during the assay at temperatures higher than 40°C (Nicolas et al., 1994). Rates of browning above pH 8.0 were not taken into consideration since rapid non-enzymatic browning of substrates may occur at higher pH values (Shannon & Pratt, 1967; Vámos-Vigyázó & Gajzágó, 1978). It was also taken into consideration that browning due to oxidation of phenols by phenolases may also involve the participation of other enzymes, may be non-enzymatic, or the browning may not involve phenols at all (Vámos-Vigyázó, 1981). Most of the previously reported assays of apple PPO have established similar limits (Mayer & Harel, 1981; Vámos-Vigyázó, 1981; Nicolas et al., 1994).
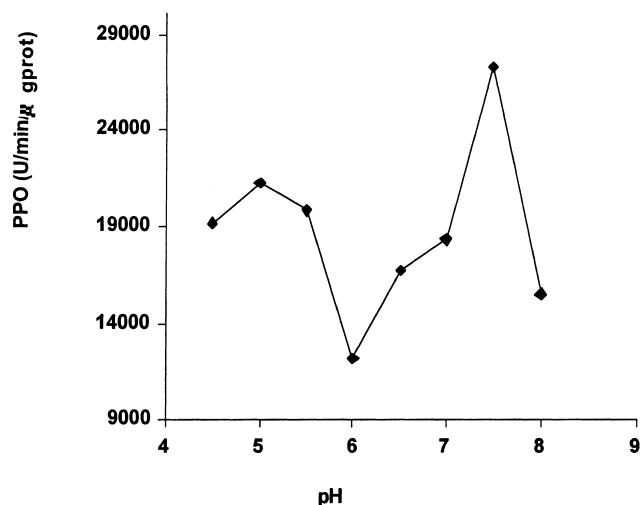

Fig. 1. pH optima for activity of 'Jonagored' apple PPO. Maximum activity with catechol was 27275 U/min/µg of protein.

The extraction pH activity curve is characterized by a steep decline between pH 5.0 and 6.0 and by an equally rapid increase from pH 7 to 7.5. The peak at pH 5.0 was lower than at pH 7.5 (Fig. 1). Mihályi, Vámos-Vigyázó, Kiss-Kutz & Babos-Szebenyi (1978) reported that 'Starking' apple exhibited a well-defined activity, maximum of PPO, at pH 6.1 and 'Jonathan' apple at pH 6.2. The increase of activity with pH was found to vary even among two different lots of the same apple variety ('Starking'), especially in the range of the highest pH (from 5.6 to 6.45). However, the maximum was observed at the same pH for both of two lots. Shannon & Pratt (1967) reported two pH optima for PPO from cv. Rome Beauty, Winesap and Cortland apples, i.e., 5.2 and 7.3, with the main maximum at the lower pH value. Mayer & Harel (1981) also reported that apple PPO had two pH optima: 5.1 for PPO from chloroplasts and 7.3 for PPO from mitochondria. They also found that Triton X100 is more effective for extracting PPO from the chloroplast than from the mitochondria. According to the optima extraction pH of 'Jonagored' PPO observed (Fig. 1), it seems to be mainly a mitochondrial enzyme. Nevertheless, as it had another maximum of activity at pH 5.0, it may also contain an isoenzyme of chloroplasmic origin (Vámos-Vigyázó, 1981). Additionally, detergents may affect either the cytoplasm or the mitochondria enzyme. Subsequent studies should be performed if the PPO origin is intended to be clarified.

3.3. Kinetic parameters

V_{max} and K_m for apple PPO activity for different substrates are in Table 3. The enzyme seemed to have the highest affinity (lowest K_m value) for L-dopa, 4-methylcatechol and (+) catechin. However, considering the ratio V_{max}/K_m, p-cresol was found to be the most

Table 3
Michaelis–Menten constant (K_m) and maximum activity (V_{max}) of apple PPO on different substrates

Substrates[a]	Wave length (λ)	V_{max} (U/min)	K_m (mM)	V_{max}/K_m (U/mM/min)
Diphenols				
Catechol	420	1100	230	4.78
(+) catechin	420	340.1	13	26.2
L-dopa	480	344.8	10	34.5
Dopamine	480	1196	59	20.3
4-methylcatechol	420	405.8	12	33.8
Monophenols				
p-cresol	380	4290	33	130
L-tyrosine	420	(–)[b]	(–)	

[a] Substrates were dissolved in distilled water at pH $\cong$ 6.5.
[b] (–) The reaction did not follow Michaelis's kinetics.

efficient phenolic substrate for 'Jonagored' PPO, followed by L-dopa and 4-methylcatechol (Table 3). Tyrosine was found to be a poor substrate for the apple enzyme, which is in agreement with results from a previous study performed with 'Starking' variety (Rocha et al., 1998) and with other authors who have worked with apples (Nicolas et al., 1994) and other fruits (Benjamin & Montgomery, 1973). Richard-Forget et al. (1992) showed that several compounds such as chlorogenic acid and catechins appeared to be better substrates than 4-methylcatechol for PPO extracted from 'Red Delicious' apples. For example, they reported a ratio V_{max}/K_m of 24.8 for chlorogenic acid, 20.4 for 4-methylcatechol and 3.9 for catechol. Nevertheless, the extract was not completely devoid of monophenolase since high activity was detected toward p-cresol (Table 3).

In this study, the values of K_m for PPO from 'Jonagored' apple obtained for the substrates assayed showed a relatively low affinity of the enzyme for the substrate, since those values were overall much higher than those reported in the literature for other apple varieties: Vámos-Vigyázó & Gajzágó (1978) reported for 'Jonathan' and 'Starking' varieties K_m values, in mM, between 2.13 and 3.38 for 4-methylcatechol, 1.55 and 3.25 for chlorogenic acid, 4.30 and 5.25 for catechin and 9.3 for DOPA; Vámos-Vigyázó (1981) reported for apple K_m values, in mM, between 1.66 for chlorogenic acid, 1.48 for catechin and 4.6 for catechol; Nicolas et al. (1994) reported for apples K_m values, in mM, between 2.1 and 4.7 for 4-methylcatechol, 1.6 and 3.9 for chlorogenic acid, 1.2 and 5.8 for catechin and 5.3 and 140 for catechol; Janovitz-Klapp et al. (1989) reported for 'Red Delicious' variety K_m values of 5.2 mM for 4-methylcatechol, 4.2 mM for chlorogenic acid, 6.2 mM for catechin. However, the K_m value obtained for catechol in this study (230 mM) was quite similar to that reported by Satjawatcharaphong et al. (1983) (220 mM) for PPO extracted from 'Red Delicious' apple.

The large ranges in the apparent K_m values of PPO reported for different phenols by Nicolas et al. (1994) were not considered definitive, since they could have been due to different reasons: different assay methods used, different apple varieties, different origins of the same variety and different values of pH of extraction.

On the other hand, it is generally assumed that the pH undoubtedly affects the apparent K_m values. Janovitz-Klapp et al. (1989) showed that the apparent K_m values of 'Red Delicious' apple for 4-methylcatechol, chlorogenic acid, and (+) catechin remained almost constant between pH 3.5 and 5.0, but increased above pH 5.0. In a subsequent study, the same authors obtained K_m values of the same order of those obtained for 'Jonagored' (Table 3), for 4-methylcatechol (20.5 mM at pH 6.3), and (+) catechin (6.6 mM at pH 5.5).

3.4. Electrophoretic data

The enzyme extract contained only one protein band with relative mobility of 0.45, in spite of the two pH optima for enzyme activity, which suggested more than one isoenzyme. Rocha et al. (1998) also found only one protein band in extracts from 'Starking' apple variety. Shannon & Pratt (1967) while doing research on PPO of 'Rome Beauty', 'Winesap' and 'Cortland' apples also found two pH optima for enzyme activity, but the paper electrophoresis revealed only one fraction for every variety, as in the present study.

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