Characterisation of 'Starking' Apple Polyphenoloxidase

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Abstract: Experiments were performed to optimise the extraction conditions of 'Starking' apple fruit polyphenoloxidase (PPO), to evaluate the affinity and specificity towards several substrates and to study the stability of enzyme extracts from apple samples stored under different conditions. Sodium phosphate buffer (0·2 M, pH 6·5) plus 0·25% Triton X_{100} and 1% or 2% PVPP was found to be the most efficient extraction medium. Chlorogenic acid, dopamine and 4-methylcatechol showed similar specificity towards PPO, and chlorogenic acid was found to be the best substrate for the enzyme. Enzyme extracts from frozen cut apple stored at −4°C, and extracts from lyophilised apple samples stored at 4°C were more stable than extracts obtained from fresh-cut or acetone powder samples.

Key words: acetone powder; apple; freezing; lyophilisation; 'Starking'; storage

INTRODUCTION

Polyphenoloxidase (PPO) activity has been reported as the main factor involved in apple fruit browning (Zemel *et al* 1990; Trejo-Gonzalez and Soto-Valdez 1991; Amiot *et al* 1992). Varietal differences in enzyme activity and susceptibility to browning is a well known phenomenon. Although PPO has been investigated for many apple varieties, information is scarce on PPO of 'Starking' fruit (Chaves and Tomás 1975; Vamos-Vigyázó *et al* 1977; Vamos-Vigyázó and Gajzagó 1978; Satjawatcharaphong *et al* 1983; Janovitz-Klapp *et al* 1989). The characterisation 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 to be achieved.

Two main problems are found in optimization of the extraction conditions for 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 X_{100} (Galeazzi *et al* 1981; Wesche-Ebeling and Montgomery 1990; Zhou *et al* 1993). For apple fruit, solubilisation may also be achieved after preparation of an acetone powder (Walker and Hulme 1966; Stlezig *et al* 1972; Zocca and Ryugo, 1975). Although this latter method avoids the use of a detergent, it also undoubtedly results in modification of enzyme properties (Nicolas *et al* 1994). 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 minimise 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 polyethyleneglycol (PEG) (Benjamin and Montgomery 1973; Park and Luh 1985), or soluble and insoluble polyvinyl pyrrolidone and polyvinilpolypyrrolidone (PVP and

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PVPP) (Galeazzi *et al* 1981; Benjamin and Montgomery 1973). 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 (Walter and Purcell 1980; Galeazzi *et al* 1981; Smith and Montgomery 1985; Wesche-Ebeling and Montgomery 1990).

However, as all plant tissues, and even different varieties of the same fruit, have different types and amounts of phenols, there is no universal method to effectively remove phenolic compounds or to inhibit browning. However, PEG (Chung *et al* 1983; Coseteng and Lee 1987), PVP (Walker and Hulme 1966; Goodenough and Lea 1979; Galeazzi *et al* 1981; Goodenough *et al* 1983; Choi *et al* 1987); cysteine (Walker and Hulme 1965; Chaves and Tomás 1975), and ascorbic acid (Harel *et al* 1966; Constantidines and Bedford 1967; Janovitz-Klapp *et al* 1989; Zhou *et al* 1993) are among the compounds that have been used for these purposes in crude enzymatic extracts of apple.

PPO isoenzymes isolated from higher plants are able to oxidise a wide range of monophenols and *o*-diphenols with highly variable kinetic parameters, including 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 (Vamos-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 (Vamos-Vigyázó and Gajzágó 1978). 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, however PPO of whole apple tissue has maximum activity at pH 4·5 and 5·5 (Goodenough *et al* 1993; Choi *et al* 1987; Janovitz-Klapp *et al* 1989; Trejo-Gonzalez and Soto-Valdez 1991; Richard-Forget *et al* 1992; Zhou *et al* 1993). 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 'Starking', this work was undertaken to characterize the enzyme involved. Experiments were performed in order to optimise 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 of the enzyme toward several substrates and to study the stability of the enzyme extract at different storage temperatures (usual commercial freezing and refrigeration temperatures, −4 and +4°C).

EXPERIMENTAL

Plant material

Ripe apples (cv Starking) grown in Rio Blanco, Chile were obtained from a local fruit store in Madrid and stored at 4°C. Apple maturity was characterised in terms of soluble solids content, titratable acidity and pH.

Treatment and storage conditions

Samples preparation

In order to evaluate the influence of the method for sample preservation, samples were submitted to several pretreatments: (i) freezing with liquid nitrogen at −20°C and storage at −80°C; (ii) lyophilisation after freezing at −80°C; (iii) acetone powdering by homogenisation with pure acetone at −20°C for 1 min in an external ice bath with addition of acetone (80%) at −20°C and filtration under vacuum followed by several washings with pure cold acetone until a white powder was obtained. The powder was dried overnight and stored at −80°C. Apple samples were stored for about 3 weeks until used for PPO assays. After extraction the various extracts were stored either at −4°C or +4°C and PPO activity was assayed over 8 days.

Characterisation of apple maturity

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

Titratable acidity and pH

Apple cubes were crushed and 20–30 g of juice was diluted with 250 ml of recently boiled water. Samples (25 ml) of the prepared juice was then titrated to pH 8·1 with 0·1 M NaOH. pH was measured with a pH meter (Crison model Micro pH 2002, Crison Instruments SA, Barcelona, Spain) and a xerolyte electrode (Ingold Lot 406 -MG - DXK - 57/25). The results were expressed as mg of malic acid g^{-1} fruit fresh weight.

Soluble solids content

The soluble solids content of non-diluted juice from crushed apple cubes was determined at 20°C with a hand-held refractometer, Atago model dbx - 30. Data was expressed in °Brix.

Characterisation of *in vivo* PPO activity

Evaluation of in vivo *PPO activity*

In order to confirm the presence of PPO in apple cortex fresh-cut apple was immersed in 0·07 M catechol solution and the surface colour changes were subsequently evaluated according to the procedure of Galeazzi and

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 favourable for PPO extraction.

Extraction of PPO

Some modifications were introduced to the extraction procedure described by Galeazzi and Sgarbieri (1981). Several extraction conditions were tested for frozen apple in order to select those which maintained higher PPO activities. Optimisation of extraction condition was also performed for apple samples which had been subjected to stabilisation treatments (ie frozen at −80°C, lyophilised and acetone powder). Apple samples were homogenized in the extraction buffer with an Omni mixer (Sorvall 17106) 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 × *g* (Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant was filtered through cheesecloth and its volume determined.

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. The wavelengths used were: 420 nm for catechol, (+)catechin, 4 methylcatechol and L-tyrosine; 480 nm for L-DOPA and dopamine; 400 nm for chlorogenic acid and 380 for *p*-cresol. 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 ml^{-1} min^{-1}). A unit of enzyme activity was defined as the change of 0·001 in the absorbance value under the conditions of the assay (Galeazzi *et al* 1981; Pizzocaro *et al* 1993). When a lag phase occurred, the reaction rate was measured after the lag phase. All the determinations were performed in triplicate.

Influence of pH of extraction

Apple PPO extraction was performed using 0·2 M sodium phosphate buffer with pH from 5·5 to 7·5 (Satjawataharaphong *et al* 1983; Trejo-Gonzalez and Soto-Valdez 1991). Assay of PPO activity was performed by adding 200 µl of the enzyme extract to 2·8 ml of 0·07 M catechol (prepared in 0·05 M sodium phosphate buffer, pH 6·5). Enzyme activity was measured at 420 nm using the spectrophotometric procedure described above. Three replicates were maintained for each determination.

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 for the various substrates, with variable substrate concentrations in the standard reaction mixture, at the wavelength of maximum absorption for the corresponding chromophore. Enzyme extracts were obtained from fresh apple samples. 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 and Burk 1934).

Polyacrylamide gel electrophoresis

Extracts were obtained using the optimised conditions described previously for each sample. Apple PPO was separated into multiple forms by a modification of the polyacrylamide gel electrophoresis (PAGE) procedure described by Davis (1964). A BioRad Miniprotean II dual slab cell was used for the electrophoresis. Bisacrylamide gels (10%) were used according to Laemmli (1970), but under 'native conditions' (ie without sodium dodecyl sulphate, SDS). Gels were kept in the substrate solution for approximately 30 min, followed by repeated washes with 50% ethanol solution to eliminate excess reagent and to fix the bands by precipitation of the protein. Gels were also incubated over ≈30 min 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 (Rotatum P Select). The gels were then decolored in a solution identical to the one described above but without the colorant to allow appearance of the protein bands. Relative mobilities (RM) were then calculated.

Statistical analysis

The SAS statistical analysis system package (SAS Institute, Inc, NC, USA) was used for analyses of the data. Statistical significance was assessed by two-way analyses of variance. Significant differences ($P = 0·05$) between treatments were detected using Duncan's multiple range test.

RESULTS AND DISCUSSION

The apples were at the ripe maturity stage at which they are normally used for consumption: the soluble solids content was 13·4°Brix, the pH was 4·02 and the titratable acidity was 0·027 mg malic acid g^{-1} fruit fresh weight.

TABLE 1
Influence of extraction medium on PPO activity of fresh-cut 'Starking' apple[a]

Type of extraction medium[b]	*Ratio* (*ml* g^{-1})	*PVP* (%)	*Triton* X_{100} (%)	*PPO Activity*[c] ($U\ ml^{-1}\ min^{-1}$)
Distilled water	5	—	—	320c
Distilled water	5	1 (soluble)	—	145c
Distilled water	5	1 (insoluble)	—	135c
0·2 M Sodium phosphate buffer	5	1 (soluble)	—	68c
0·2 M Sodium phosphate buffer	5	1 (insoluble)	—	440c
0·2 M Sodium phosphate buffer	2·5	1 (insoluble)	0·25	14 250a
0·2 M Sodium phosphate buffer + 1% NaCl	2·5	1 (insoluble)	0·25	12 750a
0·2 M Sodium phosphate buffer	2·5	1 (insoluble)	—	2934b
0·2 M Sodium phosphate buffer	2·5	2 (insoluble)	0·25	14 100a

[a] Substrate was 0·16 M catechol dissolved in 0·05 M sodium phosphate buffer pH 6·5 ($\lambda = 420$ nm).
[b] pH of the extraction medium was 6·5.
[c] Mean separation in columns by Duncan's multiple range test, $P = 0{\cdot}05$.

Extraction of PPO

Fresh samples
No PPO activity was detected using either 0·05 M or 0·2 M sodium phosphate buffer alone (data not shown). Low PPO activity values were detected when distilled water was used as extraction medium (Table 1). Maximum PPO activity was obtained using 0·2 M sodium phosphate buffer, pH 6·5 with addition of 1% or 2% insoluble PVP and 0·25% Triton X_{100} at a buffer to fruit tissue ratio of 2·5 (ml g^{-1}) (Table 1). Increasing the concentration of insoluble PVP to 2% resulted in no change in the activity level. Addition of 1% (w/v) NaCl to the extraction medium slightly reduced the PPO activity (Table 1). The non-ionic detergent Triton X_{100} was useful for apple PPO extraction, probably due to disruption of chloroplast membranes, making the release of PPO easier. An increase in enzymatic activity by the use of this detergent was also reported by Harel *et al* (1965) and Stelzig *et al* (1972). When the ratio of phosphate buffer plus 1% insoluble PVP to apple sample was doubled to 5, the efficiency of the extraction was negatively affected, such that PPO activity was about 6-fold lower, probably due to increased difficulty in homogenisation and in enzyme solubilisation (Table 1).

Frozen cut apple stored at −80°C
The highest activity for PPO extraction was obtained using 0·2 M sodium phosphate buffer + 1% insoluble PVP and 0·25% Triton X_{100} at pH 6·5 at a buffer/apple sample ratio of 3 (ml g^{-1}). Reducing the ratio of the buffer to apple tissue resulted in about a six-fold reduction in PPO activity (Table 2).

Lyophilised apple
The highest activity for PPO extraction was obtained using 0·2 M sodium phosphate buffer + 1% insoluble PVP and 0·25% Triton X_{100} at pH 6·5 at a buffer/apple sample ratio of 5 (ml g^{-1}) (Table 3).

Acetone powder of apple
The highest activity for PPO extraction was obtained using 0·2 M sodium phosphate buffer + 1% insoluble PVP and 0·25% Triton X_{100} at pH 6·5 at a buffer/apple sample ratio of 100 (ml g^{-1}) (Table 4).

TABLE 2
Influence of extraction medium on PPO activity of 'Starking' apple stored at −80°C[a]

Type of extraction medium[b]	*Ratio* (*ml* g^{-1})	*PVP* (%)	*Triton* X_{100} (%)	*PPO Activity*[c] ($U\ ml^{-1}\ min^{-1}$)
Distilled water	5	—	—	40c
0·2 M Sodium phosphate buffer	5	—	—	68c
0·2 M Sodium phosphate buffer	3	1 (insoluble)	0·25	9875a
0·2 M Sodium phosphate buffer	1	1 (insoluble)	0·25	1581b

[a] Substrate was 0·16 M catechol dissolved in 0·05 M sodium phosphate buffer pH 6·5 ($\lambda = 420$ nm).
[b] pH of the extraction medium was 6·5.
[c] Mean separation in columns by Duncan's multiple range test, $P = 0{\cdot}05$.

TABLE 3
Influence of extraction medium on PPO activity of lyophilised 'Starking' apple[a]

Type of extraction medium[b]	*Ratio* ($ml\ g^{-1}$)	*PVP* (%)	*Triton* X_{100} (%)	*PPO activity*[c] ($U\ ml^{-1}\ min^{-1}$)
Distilled water	33	—	—	85d
0·2 M Sodium phosphate buffer	16	1 (insoluble)	0·25	5250b
0·2 M Sodium phosphate buffer	5	1 (insoluble)	0·25	8750a
0·2 M Sodium phosphate buffer	1·7	1 (insoluble)	0·25	1581c

[a] Substrate was 0·16 M catechol dissolved in 0·05 M sodium phosphate buffer pH 6·5 ($\lambda = 420$ nm).
[b] pH of the extraction medium was 6·5.
[c] Mean separation in columns by Duncan's multiple range test, $P = 0{\cdot}05$.

TABLE 4
Influence of extraction medium on PPO activity of acetone powder of 'Starking' apple[a]

Type of extraction medium[b]	*Ratio* ($ml\ g^{-1}$)	*PVP* (%)	*Triton* X_{100} (%)	*PPO activity*[c] ($U\ ml^{-1}\ min^{-1}$)
Distilled water	59	—	—	1840c
0·2 M Sodium phosphate buffer	12·5	1 (insoluble)	0·25	4080b
0·2 M Sodium phosphate buffer	1·7	1 (insoluble)	0·25	7750a

[a] Substrate was 0·16 M catechol dissolved in 0·05 M sodium phosphate buffer pH 6·5 ($\lambda = 420$ nm).
[b] pH of the extraction medium was 6·5.
[c] Mean separation in columns by Duncan's multiple range test, $P = 0{\cdot}05$.

Influence of pH of extraction

The pH activity profile for the oxidation of catechol by 'Starking' PPO is shown on Fig 1. 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 and Pratt 1967; Vamos-Vigyázó and Gajzágó 1978). The upper limit of the pH range was set up at pH = 8·0, by rapid autoxidation of the substrates in the alkaline region (Vamos-Vigyázó and 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 (Schwimmer 1981; Vamos-Vigyázó 1981). Most of the previously reported assays of apple PPO have established similar limits (Mayer and Harel 1981; Vamos-Vigyázó 1981; Nicolas *et al* 1994).

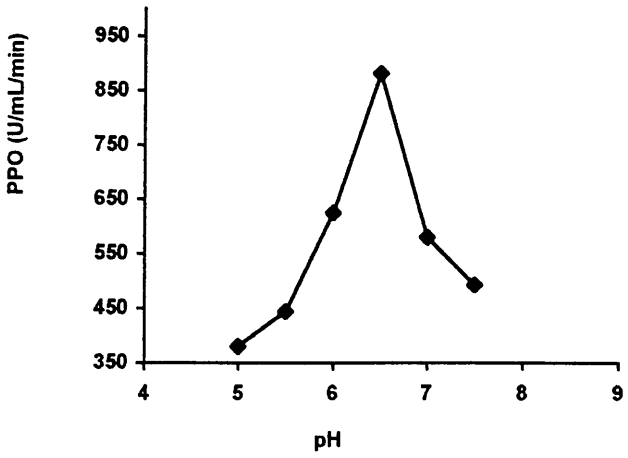

Fig 1. pH optima for activity of 'Starking' apple PPO. Maximum activity with 0·07 M catechol was 881 U ml^{-1} min^{-1}.

The pH activity curve is characterised by one peak at pH 6·5 (Fig 1), finding similar to those reported by Mihályi *et al* (1978) reported that 'Starking' apple exhibited a well-defined activity maximum of PPO at pH 6·1 and 'Jonathan' apple at pH 6·2. Aylward and Haisman (1969) reported that the optimum pH for maximum activity of PPO varies from about 4·0 to 7·0. depending upon the part of the fruit the enzyme has been extracted from, the extraction method, and the substrate. Shannon and Pratt (1967) reported two pH optima for PPO from cv Rome Beauty, Winesap and Cortland apples, ie 5·2 and 7·3, with the main maximum at the lower pH value.

Harel *et al* (1965) and Mayer and 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 X_{100} is more effective for extracting PPO from the chloroplast than from the mitochondria. According to the optima pH of 'Starking' PPO observed (Fig 1), the origin of the enzyme is not clear, it probably has a multiple origin (mitochondria and chloroplast). Subsequent studies

should be performed if the PPO origin is intended to be clarified.

Kinetic parameters

The K_m and V_{max} for apple PPO activity with different substrates are in Table 5. The enzyme seemed to have similar affinities (lowest K_m value) for chlorogenic acid, 4-methylcatechol and dopamine. However, considering the ratio V_{max}/K_{max}, *p*-cresol was found to be the most efficient phenolic substrate for 'Starking' PPO, followed by 4-methylcatechol and chlorogenic acid (Table 5). Tyrosine was found to be a poor substrate for the apple enzyme, finding which is in agreement with that of other authors who have worked with apples (Nicolas *et al* 1994), and other fruits (Benjamin and Montgomery 1973; Cash *et al* 1976; Halim and Montgomery 1978; Park *et al* 1980). Nevertheless, the extract was not completely devoid of monophenolase since high activity was detected towards *p*-cresol. Catechin and L-DOPA were not found to be good substrates for 'Starking' PPO (high K_m values), finding also reported by Vamos-Vigyázó and Gajzágó (1978) (Table 5).

In this study, the values of K_m for PPO from 'Starking' apple obtained for the substrates assayed were similar to those reported in the literature: Vamos-Vigyázó and Gajzágó (1978) reported for 'Jonathan' and 'Starking' varieties K_m in mM, values between 2·13 and 3·38 for 4-methylcatechol, 1·55 and 3·25 for chlorogenic acid; but much higher than those reported for catechin and DOPA, 4·30 and 5·25, respectively; Nicolas *et al* (1994) reported for apples values between 2·1 and 4·7 for 4-methylcatechol, 1·6 and 3·9 for chlorogenic acid and 5·3 and 140 for catechol. Janovitz-Klapp *et al* (1990) reported for 'Red Delicious' variety K_m in mM values of 5·2 for 4-methylcatechol, 4·2 for chlorogenic acid. Again, values found in this study for catechin were much higher than those reported by Janovitz-Klapp *et al* (1990) and Nicolas *et al* (1994).

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.

Electrophoretic data

The electrophoretic data on Fig 2 show that the enzyme extract contained four isoforms, using 0·16 M catechol as substrate prepared in 0·05 M sodium phosphate buffer at pH 6·5. All the extracts except acetone powder showed the presence of four isoforms with relative mobility (RM) 0·37; 0·6; 0·63 and 0·8 towards the anode. No PPO was detected from extracts of acetone powder and this may be explained by the effects of acetone on the enzyme properties (Nicolas *et al* 1994). Revealing of the gels in Coomassie Blue showed only one protein band. Shannon and Pratt (1967) while doing research on PPO of 'Rome Beauty', 'Winesap' and 'Cortland' apples also found only one fraction for all varieties, as in the present study.

Stabilisation of apple samples

When comparing apple samples prepared differently for extraction, much higher PPO activity was obtained in

TABLE 5
Michaelis–Menten constant (K_m) and maximum activity (V_{max}) for apple PPO on different substrates

Substrates[a]	*Wavelength* (λ)	V_{max} ($U\ min^{-1}$)	K_m (M)	V_{max}/K_m ($U\ mM^{-1}\ min^{-1}$)
Diphenols				
Catechol	420	0·52	0·18	2·89
(+) Catechin	420	0·77	0·085	9·06
L-DOPA	480	9·78	0·028	$3·49 \times 10^2$
Dopamine[a]	480	6·06	0·004	$1·73 \times 10^2$
Chlorogenic acid[a]	420	2·34	0·004	$6·00 \times 10^2$
4-Methylcatechol	420	2·56	0·004	$6·24 \times 10^2$
Monophenols				
p-Cresol	380	$5·79 \times 10$	0·05	$11·59 \times 10^2$
L-Tyrosine	420	—[b]	—	

[a] Substrates were dissolved in distilled water at pH ≅ 6·5.
[b] — The reaction did not follow Michaelis's kinetics.

TABLE 6
Apple PPO extract stability (U ml^{-1} min^{-1}) during 7 days of storage at two temperatures[a]

Days of storage	*Fresh-cut*		*Apple sample*				*Acetone powder*	
	4°C	*−4°C*	*Frozen (−80°C)*		*Lyophilised*		*4°C*	*−4°C*
			4°C	*−4°C*	*4°C*	*−4°C*		
0	15 500a	15 000a	74 000a	74 000a	42 000a	42 000a	55 000a	55 000a
1	14 875a	12 250b	66 000b	64 000b	42 000a	22 000c	12 500c	15 400c
2	16 250a	15 125a	65 500b	65 500b	38 500a	50 000a	12 500c	15 000c
3	14 000a	15 500a	70 500ab	65 000b	47 500a	34 750bc	22 400b	20 400b
4	11 300b	13 750a	84 000a	56 500c	33 500b	25 000c	18 600b	11 500c
7	13 125a	12 200b	56 000c	67 000a	38 000b	25 800c	20 800b	14 500c
% initial activity[b]	85	79	76	90·5	90·5	61	38	26

[a] Mean separation in columns by Duncan's multiple range test, $P = 0{\cdot}05$.
[b] Initial activity = activity on day 0.

extracts from tissue frozen and stored at −80°C (Table 6). The conditions of the extraction were 0·2 M sodium phosphate buffer (previously determined as the most efficient, Table 1) and 0·16 M catechol dissolved in 0·05 M sodium phosphate buffer at pH 6·5 as the substrate ($\lambda = 420$ nm). Tissue preparation and storage conditions of the sample before PPO extraction strongly influenced the stability of the extract (Table 6). Extracts from acetone powder lost a significant amount of activity after 1 day of storage. They retained only 38% (refrigerated extracts, 4°C) and 26% (frozen extracts, −4°C) of initial PPO activity (activity on day 0) after 7 days of storage. This may be explained by a possible interference of the acetone with the enzyme, as suggested by Nicolas *et al* (1994). Extracts from fresh-cut apple retained between 79% and 85% of their initial PPO activity (Table 6). Extracts from cut apple stored at −80°C lost more PPO activity when the extract was refrigerated (76% retention of initial activity after 7 days of storage) than when it was kept frozen (90·5%). On the other hand, extracts from lyophilized apple retained almost 90·5% of the initial PPO activity after 7 days of storage at 4°C, but retained only 61% if stored at −4°C (Table 6). Therefore, if enzymatic extracts are to be used on the day of extraction, it is best to use extracts from frozen apple stored at −4°C or extracts from lyophilised samples stored at 4°C.

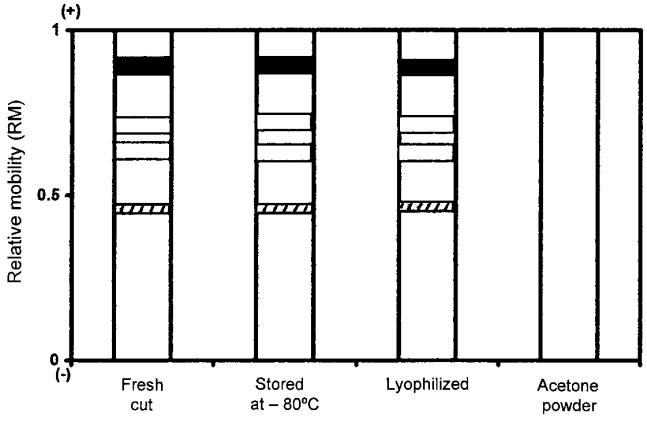

Fig 2. Electrophoretic patterns of different extracts for 'Starking' apple PPO ■, Strong; ▨, medium; □, weak.

CONCLUSIONS

In this study, a method for extraction of PPO was set up and some properties of this enzyme were determined. PPO was extracted from apples with addition of either 1 or 2% PVP and 0·25% Triton X_{100} to the extraction buffer containing phenolic adsorbents.

'Starking' apple PPO was found to have higher specificity (lower K_m) towards chlorogenic acid, 4-methylcatechol and dopamine than other phenols tested. The ratio V_{max}/K_m indicates that *p*-cresol followed by 4-methylcatechol and chlorogenic acid are the most efficient substrates for 'Starking' apple PPO. The optimum pH for 'Starking' apple PPO assays with 0·07 M catechol was 6·5. Enzymatic extracts obtained from frozen samples and stored at −80°C showed high activities and were relatively stable when stored at −4°C.

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