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Food Chemistry 92 (2005) 311–323
Food Chemistry
www.elsevier.com/locate/foodchem

Partial purification and characterisation of polyphenol oxidase and peroxidase from marula fruit (*Sclerocarya birrea* subsp. Caffra)

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Received 10 May 2004; received in revised form 21 July 2004; accepted 21 July 2004

Abstract

Marula fruit, native to sub-Saharan Africa, is of growing commercially importance. Polyphenol oxidase (PPO) and peroxidase from the fruit were partially purified by a combination of temperature induced phase separation in Triton X-114, DEAE-ion exchange and Sephadex G100 gel filtration. PPO activity was purified 58-fold with 75% recovery while the purification factor for peroxidase was 19% with 25% recovery. The enzymes were characterised for enzyme concentration–reaction rate relationship, thermal stability, pH activity and stability, molecular weight, isoelectric point (pI) and kinetic parameters. PPO and peroxidase shared the same molecular weight (71 kDa) and pI (5.43). Thermal deactivation curves were bi-phasic for both activities. Peroxidase displayed maximal activity at pH 4.0 with ABTS (2,2′-azino-(bis-3-ethylbenzthiazoline-6-sulfonic acid)) and a K_M of 1.77 mM for hydrogen peroxide. The pH optimum for PPO was 7.0 with catechol. Marula PPO had K_M values of 1.41, 1.43, 3.73 and 4.99 mM for catechin, 4-methylcatechol, 3,4-dihydroxyphenylpropanoic acid (DHPPA) and catechol, respectively.

Keywords: Enzymatic browning; Marula fruit; Polyphenol oxidase; Peroxidase; Phase separation

1. Introduction

Post-harvest changes account for over 50% of losses of fruits and vegetables worldwide (Martinez & Whitaker, 1995). Enzymatic browning is a major contributor to these losses. Polyphenol oxidase (E.C.1.14.18.1) is the principal enzyme involved in enzymatic browning. Due to its undesirable effects on fruits and vegetables, the catecholase activity (E.C.1.10.3.2) of the enzyme has been isolated and characterised from most produce of commercial importance. The properties of the enzyme have been reviewed extensively by Mayer and Harel (1979); Vamos-Vigyazo (1981); Zawistowski, Biliaderis, and Eskin (1991) and, more recently, by Yoruk and Marshall (2003).

Peroxidase (E.C 1.11.1.7) is another oxidoreductase that may be involved in enzymatic browning. Considerable attention has been devoted to the enzyme, due to its use as a heat treatment efficacy indicator as well as its role in quality deterioration (Burnette, 1977). Peroxidase may be involved in enzymatic browning since diphenols may function as reducing substrates in the peroxidase reaction (Robinson, 1991). However, the involvement of peroxidase in browning might be limited by the availability of hydrogen peroxide. Nevertheless, reports describing this involvement include Finger (1994); Jiang and Miles (1993); Lagrimini (1991); Laukkanen, Haggman, Kontunen-Soppela, and Hohtola (1999); Lopez-Serrano and Ros-Barcelo (1996); Richard-Forget and Gauillard (1997); Subramanian, Ventakesh, Ganguli, and Sinkar (1999); Underhill and Critchley (1995).

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doi:10.1016/j.foodchem.2004.07.026

Marula fruit is of growing commercial importance (Gous, Weinert, & van Wyk, 1988; Shackleton, 1996). Evidently, neither PPO or peroxidase has been isolated from marula fruit (*Sclerocarya birrea*). The effects of endogenous antioxidants on marula PPO activity have been previously studied (Mdluli & Owusu-Apenten, 2003). As a continuation of enzymatic browning studies in marula fruit, we describe the isolation and characterisation of PPO and peroxidase from the marula fruit.

2. Materials and methods

2.1. Materials

Glacial acetic acid, hydrochloric acid (37% v/v) and trichloroacetic acid were obtained from Fischer Chemicals (UK). 3,4-Dihydroxyphenylpropanoic acid (DHPPA) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from Fluka Chemicals (Dorset, UK). Polybuffer exchanger (PBE 94) for chromatofocussing and Sephadex G100 gel filtration resin were purchased from Pharmacia Biotech (Sweden). 2,2′-Azino-(bis-3-ethylbenzthiazoline-6-sulfonic acid) di-ammonium salt (ABTS), BCA protein assay kit, catechol, citric acid, dibasic potassium phosphate, gel filtration molecular weight markers (MW-GF-200 kit), hydrogen peroxide (30% v/v), imidazole, L-ascorbic acid, 4-methylcatechol (MC), monobasic potassium phosphate, and Triton X-114 were all purchased from Sigma–Aldrich (UK). Supelco (UK) supplied Toyopearl diethyl-amino-ethyl (DEAE) ion-exchange resin. All materials were of Analar grade unless otherwise stated.

2.2. Extraction of juice and preparation of crude enzyme extract

Extraction of fruit juice and preparation were performed as described elsewhere (Mdluli & Owusu-Apenten, 2003) with the exception that 10 mM phosphate buffer (pH 7.0) was used as extraction and dialysis buffer.

2.3. Triton X-114 phase separation

TX-114 was purified according to the method of Bordier (1981). The detergent layer from three phase separations was used for experiments. TX-114, a highly viscous liquid, was added to crude PPO solution to a concentration of 4% (w/v). The solution was incubated at 35 °C for 10 min. At the end of the incubation period, two distinct layers of solutions were observed. The solution was centrifuged for 10 min at 4000*g* at room temperature. The upper (aqueous) layer was dialysed overnight against 10 mM phosphate buffer (pH 7.0) and assayed for PPO and peroxidase activities.

2.4. Ion-exchange chromatography on DEAE-Toyopearl resin

The phase-separated and dialysed extract was loaded onto a DEAE-Toyopearl anion-exchange column (1.6×25) cm at a flow rate of 30 ml per hour. The column was then washed with 5–6 column volumes of 10 mM phosphate buffer (pH 7.0). Enzyme activity was eluted with a linear gradient of 10–200 mM phosphate buffer. Fractions of 10 ml were collected and assayed for PPO and peroxidase activities.

2.5. Gel filtration

Fractions showing PPO or peroxidase enzyme activity from the ion exchange isolation step were pooled and concentrated by ultra filtration using an Amicon PM 10 membrane (molecular weight cut off point of 10 kDa). The concentrated enzyme (3 ml) was applied to a Sephadex G100 column (1.6×90) cm and eluted with 100 mM phosphate buffer (pH 7.0) at a flow rate of 12 ml per hour. Fractions of 6 ml were collected and assayed for PPO or peroxidase activity.

Following the isolation procedures described above, aliquots showing either PPO or peroxidase activity were pooled and concentrated by ultra filtration. These concentrated fractions were used for the characterisation studies described below.

2.6. Enzyme assays

Polyphenol oxidase (catecholase) activity was assayed by the MBTH–quinone coupling method of Espin, Morales, Varon, Tudela, and Garcia-Canovas (1996). Using catechol (30 mM) and MBTH (0.33 mM) as substrate and quinone coupler, respectively, initial rates of MBTH–quinone adduct formation were measured on a Cecil 7200 double beam spectrophotometer fitted with a thermostatted cuvette (37 °C), at 500 nm. The initial rate was calculated from the slope of the absorbance–time curve. Total reaction volume was always maintained at 3.0 ml. One unit of enzyme was defined as a 0.001 change in absorbance per minute. The effect of adding partially purified PPO on reaction rates was noted. Assays were performed at pH 6.5 or 7.0.

Peroxidase assays were done with ABTS (2 mM) and H_2O_2 (0.8 mM) in citrate–phosphate buffer. Initial rates of ABTS free radical formation were monitored at 414 nm. The effect of varying added enzyme on rate of free radical formation was studied. Assays were performed at pH 3.5 or 4.0. One unit of enzyme was defined as 0.001 change in absorbance per minute.

2.7. pH stability profile

The partially purified enzymes were originally isolated in 100 mM phosphate buffer (pH 7.0). It was necessary to change this buffer. Therefore, the enzyme was ultrafiltered and then re-suspended in 5 mM phosphate buffer (pH 7.0) to a desired volume. Samples were mixed with 3 volumes of citrate phosphate buffer at the appropriate pH, ranging from 3.0 to 7.0 at one pH unit intervals. Phosphate buffer (50 mM) was used at pH values of 7.0 and 8.0. After mixing of the sample and buffer, the pH was re-checked. PPO activities from samples were incubated at 37 °C for 30 min prior to the assay for residual activity at a fixed pH 7.0. Peroxidase samples were incubated at 25 °C for the same duration before being assayed for residual activity at pH 4.0 at the same temperature.

2.8. pH-activity profile for partially purified marula fruit enzymes

Studies were performed with 100 mM citrate–200 mM phosphate buffer (pH 3.0–7.0) and phosphate buffer (100 mM) at pH 7.0 and 8.0. Enzyme volume was kept constant throughout this study. The effect of pH on partially purified marula fruit PPO activity was determined with catechol (20 mM).

The pH activity profile for partially purified peroxidase was performed with ABTS (2mM) and hydrogen peroxide (0.8 mM).

2.9. Substrate specificity studies

Partially purified marula PPO. Four substrates were used at various concentrations, depending on their solubility; 2.5, 5, 10, 20, and 40 mM for catechol, MC, and DHPPA; 1.04, 2.08, 4, and 10 mM for catechin. Substrates were dissolved in 100 mM phosphate buffer, pH 7.0. At each substrate concentration, the initial rate of MBTH–quinone adduct formation was monitored. Apparent kinetic parameters (K_M and V_{max}) were determined using the Lineweaver–Burk double reciprocal plot.

Partially purified marula peroxidase. Kinetic parameters (K_M and V_{max}) were determined with ABTS and hydrogen peroxide. The concentrations of hydrogen peroxide used were 0.2, 0.4, 0.6, 0.8 and 1 mM at constant ABTS concentrations of either 2, 4, or 8 mM. At each ABTS concentration, the hydrogen peroxide concentration was varied over the range specified. Kinetic parameters were determined using the Lineweaver–Burk double reciprocal plot. From the re-plots of $1/K_M$ (for hydrogen peroxide) versus the reciprocal of ABTS concentration, the "true" kinetic parameters of K_M and V_{max} were determined for both substrates.

2.10. Thermal inactivation of partially purified marula fruit polyphenol oxidase

Buffer solution (2 ml, 100 mM phosphate buffer, pH 7.0) in thin-walled glass sample bottles was pre-heated at 60, 70, 80 and 90 °C in a water bath for 7– 8 min, after which enzyme solution was added (1 ml). Samples were taken after 1, 2, 4, 8, 16 min for enzyme incubated at 60, 70 and 80 °C. For studies at 90 °C, no sample was taken at 16 min. Samples were rapidly cooled by immersing them, immediately after withdrawal, in an ice bath. After 10 min, the remaining enzyme (PPO and peroxidase) activity was assayed. The residual activity was calculated as a percentage of unheated enzyme.

2.11. Molecular weight determination

The molecular weight of marula fruit PPO activity was determined using a calibrated Sephadex G100 (1.6×90) cm column. The column was calibrated using a commercial kit (Sigma MW-GF-200 kit) containing five proteins of known molecular weight. The samples used for molecular weight determination were from the ion-exchange fractionated marula fruit enzymes.

2.12. Determination of isoelectric point

The isoelectric point of marula fruit enzymes was determined by chromatofocussing on a PBE 94 column (1×20) cm. The column was equilibrated with 25 mM imidazole buffer, pH 7.4. Following DEAE anion-exchange chromatography, fractions showing PPO or peroxidase activity were pooled and concentrated by ultrafiltration. Phosphate buffer, in which the enzymes were dissolved, was exchanged for imidazole (25 mM pH 7.4) by ultrafiltration. The sample was loaded onto the column. Polybuffer 74, adjusted to pH 4.0 with 20 mM hydrochloric acid, was used to elute the enzyme activity. The elution of the sample was monitored by $A280$ nm readings. Eluted sample was collected in 5 ml aliquots and the pH was measured as soon as possible after elution. This was followed by an assay for PPO and peroxidase activity to determine the location of the enzyme in different fractions.

2.13. Protein determination

Protein content was quantified using the micro-assay BCA protein determination kit (Sigma) with bovine serum albumin (BSA) as standard.

3. Results and discussion

3.1. Purification

Fig. 1 shows the IEC elution profiles (absorbance at 280 nm and enzyme activity) for marula fruit PPO and peroxidase. Significant features of the enzyme profiles include the co-elution of marula PPO and peroxidase as well as the presence of two forms for both enzymes – a small amount of an unadsorbed form elutes during the washing stage and a bound form of the enzymes that eluted with 38 mM phosphate buffer. Similar enzyme elution profiles were observed when experiments were performed at pH 6.0 and 8.0 (data not shown). The *A*280 nm elution profile (Fig. 1) was characterised by three peaks, two of which were associated with PPO and peroxidase activity. No enzyme activity was associated with the second major *A*280 peak. Subsequent determination of protein content by the BCA method, for all three peaks, showed the presence of protein in the first and third peaks but not the second peak. Thus, while the relative absorbance of the material in the second peak was high, the lack of protein content suggests that this material was not protein.

The *A*280 nm and enzyme elution profiles from gel filtration are shown in Fig. 2. The enzyme elution profile shows a single peak for PPO activity. Peroxidase also shows a single peak of enzyme activity. As with IEC, marula PPO co-elutes with peroxidase on gel filtration. The co-elution of PPO and peroxidase is not novel; De Biasi and Badiani (1990); Okpuzor and Omidjii (1998); Sheen and Calvert (1969); Srivastava and van Huystee (1977); Tremolieres and Bieth (1984) have described the same phenomenon.

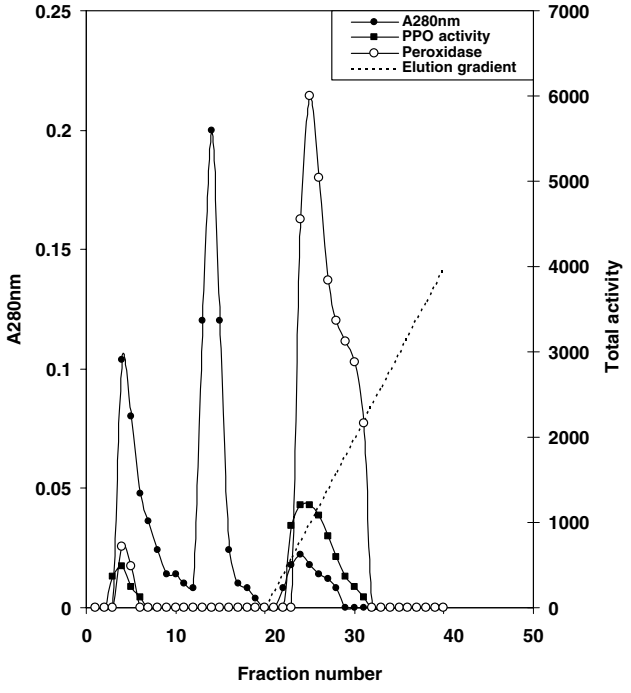

Fig. 1. Ion exchange profiles for marula peroxidase and polyphenol oxidase dialysed phase-fractionated sample was loaded onto DEAE column. The column was then washed with 200 ml of 10 mM-phosphate buffer, pH 7.0. Enzyme activity was eluted with linear gradient of 0–200 mM phosphate buffer, pH 7.0.

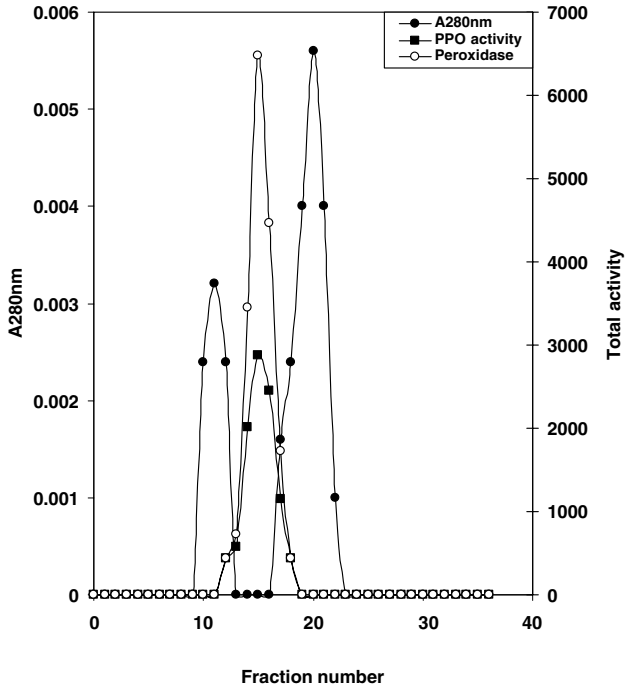

Fig. 2. Gel filtration elution profiles for marula peroxidase and polyphenol oxidase. Samples with POD and PPO activity, following ion exchange chromatography, were pooled and concentrated by ultrafiltration. The concentrated sample was loaded onto a Sephadex G100 gel filtration column. Column was eluted with 100 mM-phosphate buffer.

Polyphenolic compounds interfere with purification of proteins from plants; their multidentate ligand properties enable them to cross-link protein via hydrogen bonding and covalent interactions (Sanchez-Ferrer, Bru, & Garcia-Carmona, 1994). Moreover, tissue homogenisation during enzyme isolation initiates browning reactions whose intermediates (quinones) may also form covalent linkages that may not be reversible. The undesirable effects of polyphenolic compounds are routinely minimised by measures that may include tissue homogenisation in a nitrogen atmosphere, at low temperatures, and the addition of phenol-absorbing polymers, such as polyethylene glycol (PEG) or PVP (Vamos-Vigyazo, 1981). Addition of reducing agents, such as ascorbate and sulphite, is also undertaken to minimise protein phenolic interactions (Mayer & Harel, 1979). With respect to isolation of PPO, once the problems of phenol/quinone interaction with PPO are overcome, ammonium sulphate fractionation or acetone precipitation is regularly used as the first step of an

isolation strategy. Enzyme isolation by either of the precipitation methods is normally followed by chromatographic separation. Alternatively, temperature induced phase separation (TIPS) in the non-ionic detergent, Triton X-114 (Bordier, 1981) has been used in place of protein precipitation methods described above for isolation of PPO. Besides grape PPO (Sanchez-Ferrer, Bru, & Garcia-Carmona, 1989), TIPS has been applied in the isolation of PPO from, broad bean leaf (Sanchez-Ferrer, Bru, & Garcia-Carmona, 1990), fruits of prunus (Fragnier, Marques, Fleuriet, & Macheix, 1995), verdedoncella apple (Espin, Morales, Varon, Tudela, & Garcia-Canovas, 1995), iceberg lettuce (Chazarra, Cabanes, Escribano, & Garcia-Carmona, 1996), mushroom (Nunez-Delicado, Bru, Sanchez-Ferrer, & Garcia-Carmona, 1996), table beet (Escribano, Cabanes, & Garcia-Carmona, 1997), blanquilla pear (Espin, Morales, Varon, Tudela, & Garcia-Canovas, 1997), banana (Mar-Sojo, Nunez-Delicado, Garcia-Carmona, & Sanchez-Ferrer, 1998), desert truffle (Perez-Gilabert, Morte, Honrubia, & Garcia-Carmona, 2001) and persimmon fruit (Ozen, Colak, Dincer, & Guner, 2004). The relative merits of TIPS in place of the more traditional techniques are discussed extensively by Sanchez-Ferrer et al. (1994).

The effect of TX-114 phase separation on marula PPO and peroxidase activity is shown in Table 1. TX-114 temperature induced phase partitioning (TIPS) results in a threefold purification for PPO.

Latent forms of PPO are known to be activated by a number of agents, including detergents, temperature and pH. The possibility of activation of PPO by exposure to the temperature (35 °C) at which phase separation was carried out was investigated by exposing a sample to that temperature for the same duration of time as that required for phase separation. A sample exposed to 35C had a total activity of 14,090 Units (103% of crude extract). The results show that marula fruit PPO is not activated by exposure to the phase separation temperature. Marula fruit PPO activity increased 58-fold after TIPS, IEC and gel filtration (Table 1).

The utilisation of TIPS in the purification of peroxidase does not appear to be as widespread as its use in PPO purification. TIPS resulted in a threefold purification of marula peroxidase. A purification of fivefold has been reported for peroxidase from sago palm (Onsa, Saari, Selamat, & Bakar, 2004).

It is noteworthy that the recovery of enzyme activity following the TIPS is extremely high-153% and 203% for marula PPO and peroxidase, respectively, of the original activity. The high recovery suggests that TIPS removes inhibitors that interfere with enzyme activity.

Using the purification scheme discussed (TIPS, DEAE ion exchange and gel filtration) marula PPO was purified 58-fold with 75% enzyme recovery while the purification factor for peroxidase was 19 and enzyme recovery of 25%. For marula peroxidase, gel filtration led to a large loss of enzyme activity – about 66% of the activity applied to the Sephadex G100 column was not recovered. This suggests that size exclusion chromatography removed some low molecular weight component necessary for activity. Marula PPO and peroxidase were not purified to homogeneity as multiple bands were observed on silver-stained SDS–PAGE of enzyme samples obtained from the final isolation step (data not shown).

3.2. Characterisation of partially purified marula fruit PPO and peroxidase

The effect of varying the volume of partially purified marula fruit PPO and peroxidase on respective initial reaction rates is shown in Fig. 3. Initial rates of PPO-catalysed catechol oxidation increased with increased volume of added enzyme. The linear regression equation for the PPO-catalysed reaction was $y = 0.4884x$ ($R^2 = 0.9929$). Previous studies with crude extracts of marula

Table 1
Effects of phase separation, ion exchange and gel filtration chromatography on marula PPO and peroxidase activity

	Total activity (Units)	Activity recovery (%)	Total protein (mg)	Protein yield (%)	Specific activity U mg^{-1}	Fold purification
Marula PPO						
Crude[a]	13,260	100	3.8	100	3489	1
TIPS[b]	20,280	153	2.1	55	9657	3.0
DEAE[c]	15,600	118	0.28	9	55,714	16
GF[d]	9936	75	0.05	1.3	202,776	58
Peroxidase						
Crude	70,980	100	3.8	100	18,679	1
TIPS	144,300	203	2.1	55	68,714	3.7
DEAE	52,320	73	0.28	9	186,857	10
GF	17,712	25	0.05	1.3	361,469	19

[a] Freeze-dried juice dissolved in phosphate buffer, centrifuged and dialysed overnight against phosphate buffer (10 mM).
[b] Sample phase separated in 4% (w/v) TX-114, centrifuged and dialysed overnight.
[c] Dialysed phase-separated sample loaded on to ion exchange chromatography column.
[d] Gel filtration of pooled fractions showing enzyme activity from ion-exchange chromatography.

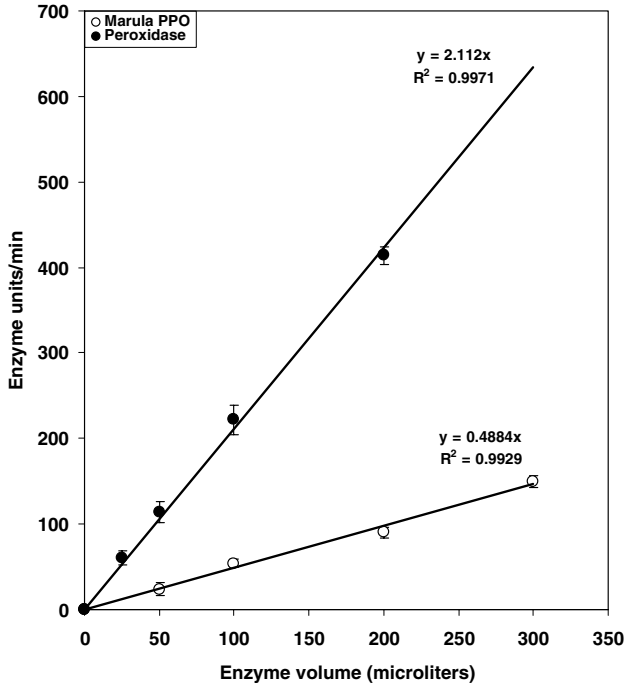

Fig. 3. Effect of enzyme volume on reaction rates PPO assayed with 30 mM catechol and 0.33 mM MBTH. MBTH–quinone adduct formation was monitored at 500 nm at 37 °C in 100 mM phosphate buffer, pH 7. Peroxidase assay was performed with ABTS (2 mM), H_2O_2 (0.8 mM) in citrate phosphate buffer, pH 3.5 at 25 °C. Initial rates of ABTS radical formation were monitored at 414 nm. Values are averages ± SD, $n = 3$.

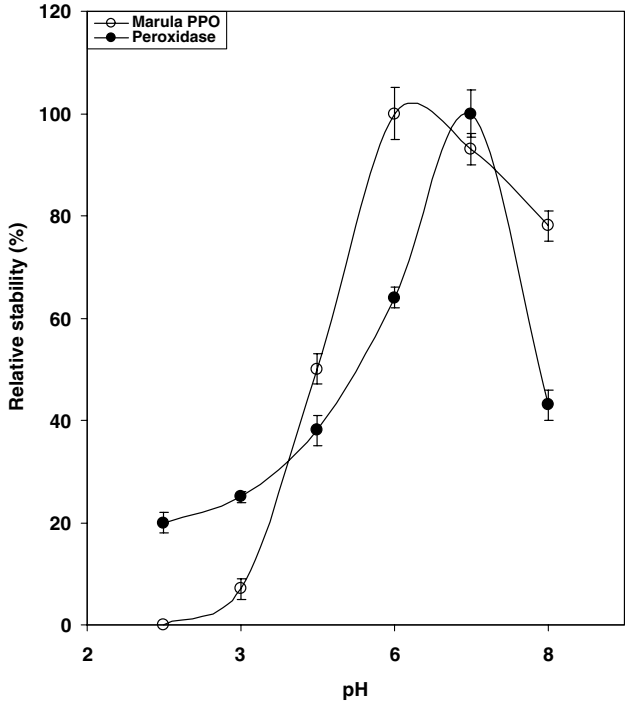

Fig. 4. pH-stability profiles of marula polyphenol oxidase and peroxidase. Marula polyphenol oxidase samples were incubated at pH values shown in the graph for 30 min at 37 °C. Residual PPO activity was assayed at pH 7.0 with catechol (30 mM) and MBTH (0.33 mM) at 37 °C. Peroxidase samples were incubated at appropriate pH for 30 min at 25 °C before being assayed for residual activity at pH 3.5 (25 °C) with ABTS (2 mM) and H_2O_2 (0.8 mM). Values are averages ± SD, $n = 3$.

fruits showed that the activity was associated with significant lag times that interfered with attempts to establish the reaction rate-enzyme concentration relationship (Mdluli & Owusu-Apenten, 2003). No lag times associated with partially purified marula PPO were observed. ABTS free radical formation increased linearly ($y = 2.11x$, $R^2 = 0.9971$) with increased volume of added partially purified peroxidase.

3.3. pH stability

Fig. 4 shows the pH-stability profile for the partially purified enzymes. Partially purified marula PPO was found to be most stable at pH 6.0 while, at more acidic pH values, it was unstable with no activity being detected after exposure to pH 3.0. Partially purified marula peroxidase was most stable at pH 7.0. Above and below this pH value the enzyme appears to be less stable.

3.4. pH activity profile

The apparent pH optima for purified marula enzymes are shown in Fig. 5. Marula fruit PPO has a pH optimum of 7.0 with catechol as substrate. This compared favourably with a pH optimum of pH 6.5 for crude marula fruit PPO (Mdluli, 2001). The pH optimum for both crude and purified marula PPO is within the range (pH 4.0–7.0) in which most PPOs display optimum activity (Vamos-Vigyazo, 1981). The low activity observed at more acidic pH values may be due to enzyme instability at these pH values (Fig. 4).

The pH optimum for purified marula peroxidase was found to be pH 4.0 with ABTS as substrate. This value compares favourably with that observed for the crude enzyme (pH 3.5) (Mdluli, 2001). Enzyme activity declines rapidly as the pH approaches 7.0. This lack of activity at higher pH values is not due to enzyme instability, since Fig. 4 shows that marula peroxidase is most stable at pH 7.0. McCoy-Messer and Bateman (1993) have described the instability of the ABTS radical at relatively high pH values and in biological buffers. However, the relative instability of the radical may be discounted for the lack of activity observed in this study because no biological buffers were used in this work. Moreover, the half-life of the radical at the pH values and in the buffer used in this work is in excess of the assay time. The half life of the radical at pH 7.5, in potassium phosphate buffer, was reported to be 79 min while the total time taken to assay was 5–6 min.

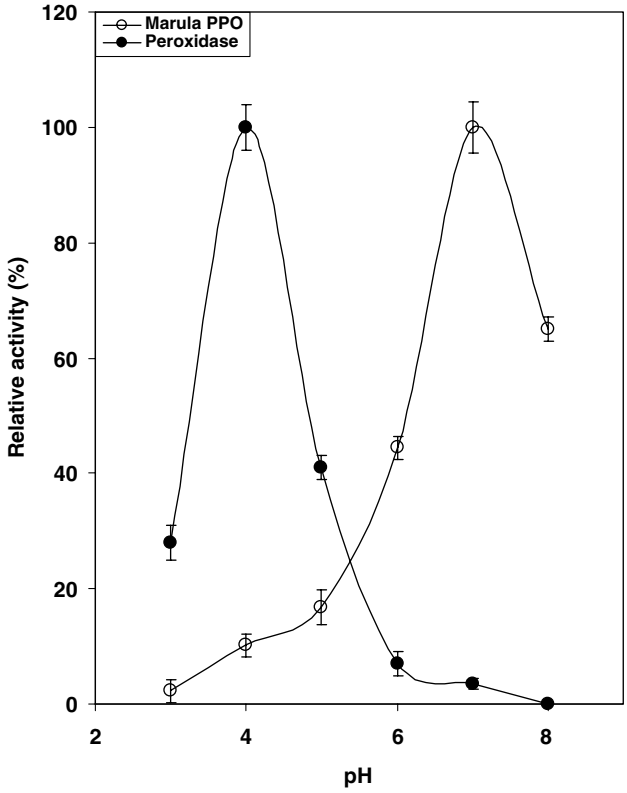

Fig. 5. pH-activity profiles for marula polyphenol oxidase and peroxidase. Substrates were prepared at pH values indicated on the graph. PPO activity was determined with catechol (30 mM) and MBTH (0.33 mM) at 37 °C. Peroxidase assay was performed with ABTS (2 mM) and H_2O_2 (0.8 mM) at 25 °C. Values are averages ± standard deviation, $n = 3$.

3.5. Substrate specificity

The apparent kinetic parameters for partially purified marula fruit PPO are shown in Table 2. The Lineweaver–Burk plots, from which the kinetic parameters were derived, are shown in Fig. 6. The plots shown are for catechol, DHPPA and MC. The K_M values for marula PPO, in order of decreasing affinity were 1.41, 1.45, 3.73 and 4.99 mM, for catechin, MC, DHPPA and catechol, respectively. However, using V_{max}/K_M as the criterion for catalytic efficiency, the order of suitability as substrate for marula fruit PPO is MC > catechin > catechol > DHPPA. This finding of MC as the best substrate for marula fruit PPO is similar to that found for numerous plant PPOs.

Marula peroxidase kinetic parameters (K_M and V_{max}), determined with ABTS fixed at concentrations of 2, 4, or 8 mM, are shown in Table 3. The concentration of hydrogen peroxide was varied, as shown in Fig. 7. Fig. 7 illustrates the Lineweaver–Burk double reciprocal plots from these studies. The parallel lines in Fig. 7 are consistent with a Ping-Pong Bi Bi mechanism for a peroxidase. Both K_M and V_{max} varied as a function of the concentration of ABTS. The "true" parameters can be calculated from re-plots of the reciprocal of the apparent V_{max} versus the reciprocal of the reducing substrate (ABTS) and apparent K_M versus the reciprocal of the reducing substrate. Figs. 8 and 9 show the secondary plot for the determination of V_{max} and K_M. The K_M values for marula peroxidase were 9.56 and 1.77 mM, for ABTS and hydrogen peroxide, respectively. Other reported K_M values for hydrogen peroxide include 0.85 mM for one turnip isoenzyme (Duarte-Vazquez, Garcia-Almendarez, Regalado, & Whitaker, 2000), 11.4 and 6.2 mM for Brussels sprouts isoenzymes (Regalado, Perez-Arvizu, Garcia-Almendarez, & Whitaker, 1999), 1.5 mM for pear (Richard-Forget & Gauillard, 1997) and 13.6 mM for Araucaria seeds enzyme (Riquelme & Cardemil, 1993).

3.6. Thermal inactivation kinetics

The thermal inactivation profiles of partially purified marula fruit PPO and peroxidase are shown in Figs. 10 and 11, respectively. In both instances, the profiles are biphasic and characterised by a rapid decline in activity on exposure to heat, followed by a more gradual decrease in activity on continued exposure. This biphasic behaviour is more pronounced at 80 and 90 °C than at 60 and 70 °C for both enzymes. At 60 °C, marula fruit PPO is relatively heat-stable, retaining up to 60% activity after 16 min of heating, while 70% activity is retained for peroxidase at the same temperature.

The rate constants for thermal inactivation k, for the first phase of thermal inactivation were calculated from the slope of the curve at each temperature. The temperature dependence of k was evaluated using the Arrhenius equation:

Table 2
Summary of kinetic parameters for partially purified polyphenol oxidase

Substrate[a]	K_M (mM)	V_{max} U mg^{-1} Protein × 10^3	V_{max}/K_M U mg^{-1} Protein/mM × 10^3
Catechol	4.99	122	24.2
4 MC	1.45	69.5	47.9
DHPPA	3.73	75.1	20.1
(+)-Catechin	1.41	54.2	38.5

[a] All parameters were determined at pH 7.0 using 100 mM phosphate buffer. MBTH assay used to monitor product formation at 500 nm (catechol and DHPPA), 494 nm (4-MC) and 458 nm (catechin).

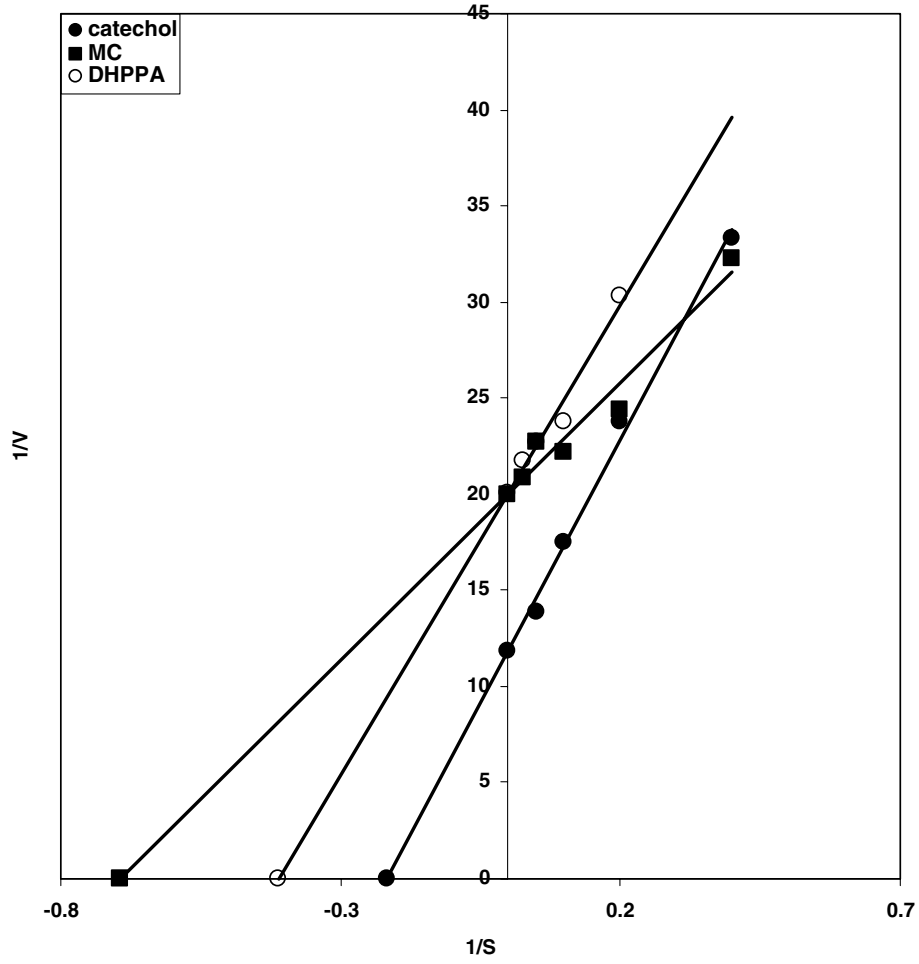

Fig. 6. Lineweaver–Burk plots for partially purified marula polyphenol oxidase. Enzyme assays were carried out in 100 mM-phosphate buffer (pH 7.0). MBTH–quinone adduct formation was monitored at appropriate wavelength for each substrate.

Table 3
Summary of kinetic parameters of partially purified marula peroxidase

K_M (H_2O_2) (mM)	K_M (ABTS) (mM)	V_{max}[a]	$V_{max}/K_M \times 10^5$
1.77	9.56	1.47	1.41 1.56

[a] V_{max} has dimensions of 10^6 enzyme units mg^{-1} protein.

$$\mathrm{Ln}k = C - \Delta E/RT,$$

where C is a constant of integration, ΔE is activation energy ($J\,mol^{-1}$), R (8.314 $J\,mol^{-1}$) is the universal gas constant and T (K) is the absolute temperature in Kelvin. From a plot of Ln k versus $1/T$ shown in Fig. 12, ΔE was calculated from the slope of the straight line. Other transition state parameters, namely, $\Delta G^{\#}$ (Gibbs free energy for enzyme inactivation), $\Delta H^{\#}$, enthalpy change (a measure of the number of covalent bonds broken) and $\Delta S^{\#}$, the entropy change, which is a measure of net enzyme and solvent disorder, were calculated using equations described by Forsyth, Apenten, and Robinson (1999).

The D-value or decimal reduction time (time required for 90% reduction (one $\log_{10}$ cycle) in activity at a particular temperature) and the half-life ($t1/2$) (time required for 50% reduction in activity) were calculated from the following equations:

$$D\text{-value} = \mathrm{Ln}10/k,$$

$$t1/2 = \mathrm{Ln}2/\mathrm{k}.$$

The thermal inactivation parameters for PPO and peroxidase are summarised in Table 4. $\Delta H^{\#}$ approximates $\Delta E^{\#}$ (since $\Delta H^{\#} = \Delta E^{\#} - \mathrm{RT}$ and $\mathrm{RT} \approx 2.8$ $kJ\,mol^{-1}$). $\Delta H^{\#}$ and $\Delta S^{\#}$ for peroxidase are higher than those for PPO at all temperatures. In addition, D- and

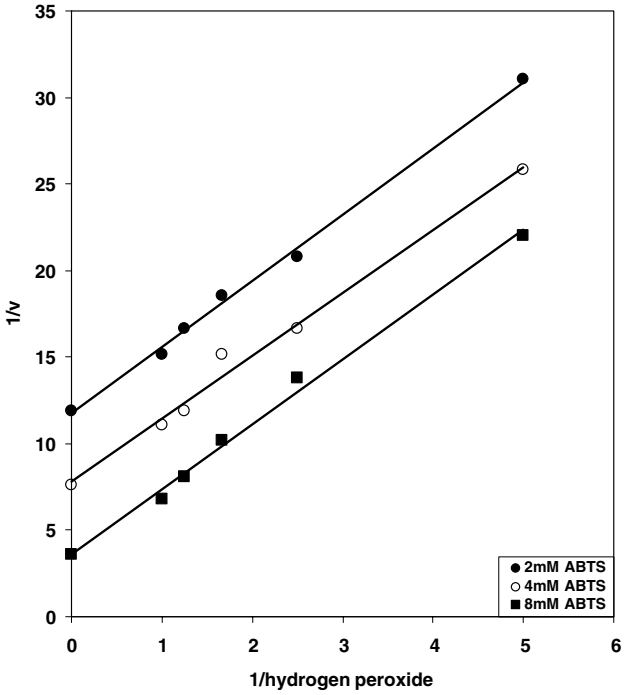

Fig. 7. Lineweaver–Burk double reciprocal plots for partially purified marula peroxidase. Primary plots are shown, in which hydrogen peroxide is used as varied substrate at three different fixed concentrations of ABTS. Enzyme concentration was kept constant.

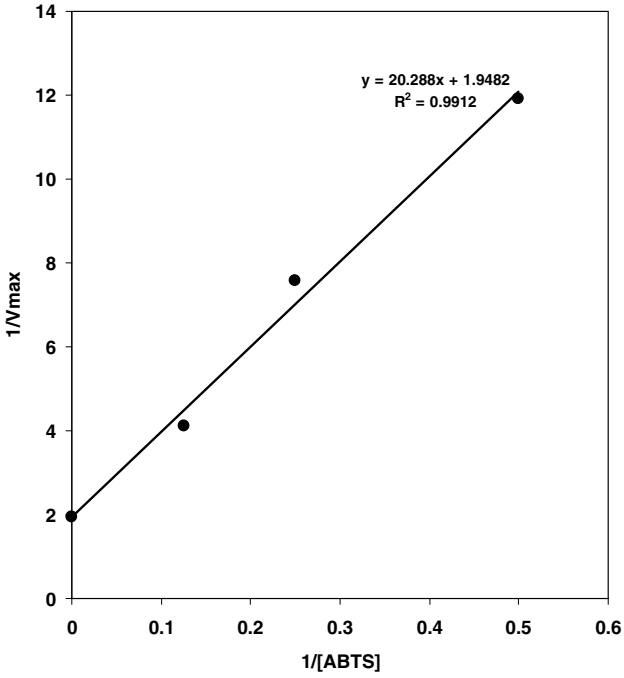

Fig. 8. Re-plot of apparent V_{max} from Fig. 7 vs.1/ABTS concentration y-axis shows the apparent $1/V_{max}$ values for hydrogen peroxide.

half-life values for peroxidase are greater than those for PPO at 60 and 70 °C. The results suggest that marula peroxidase is more thermostable than PPO, particularly at the lower temperatures used in the study.

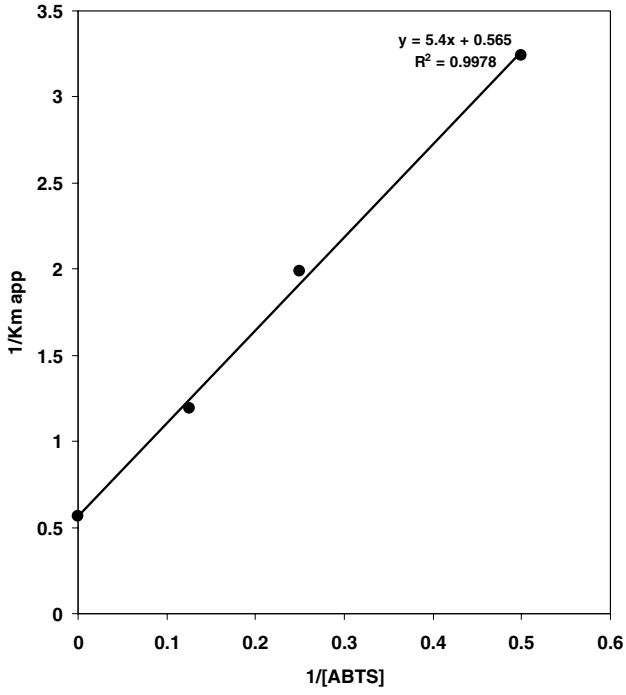

Fig. 9. Re-plot of apparent K_M from Fig. 5 vs. 1/ABTS concentration y-axis shows $1/K_M$. K_M values are the apparent values for hydrogen peroxide.

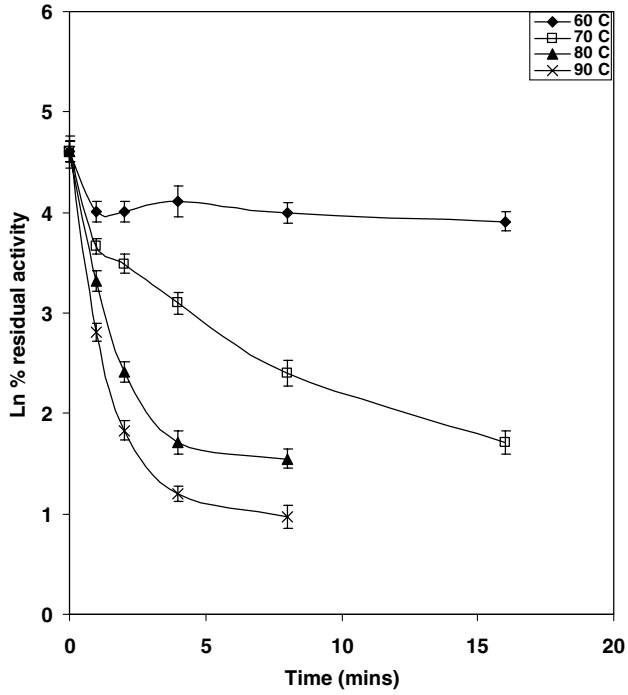

Fig. 10. Heat-inactivation profile for partially purified marula polyphenol oxidase. Thermal treatment carried out at temperatures shown in the graph. Control and residual activity assayed with 30 mM catechol (0.33 mM MBTH) in 100 mM-phosphate buffer, pH 7.0, at 37 °C. Values are averages ± SD, $n = 3$.

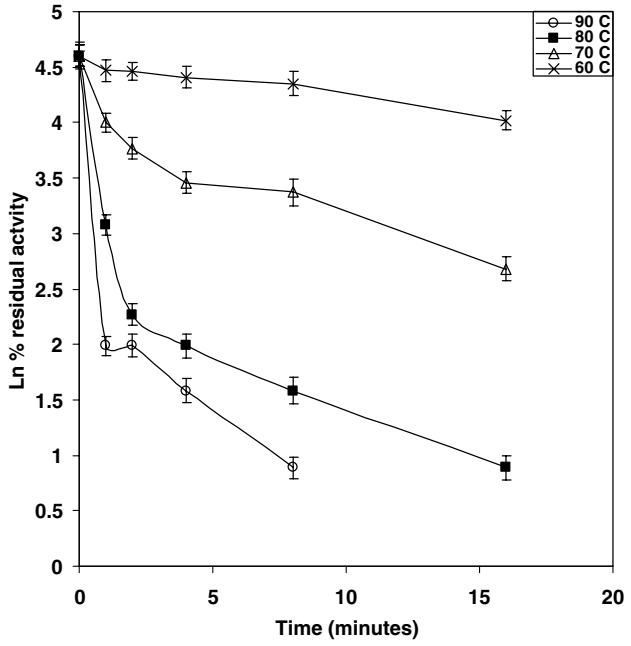

Fig. 11. Thermal inactiviation profile for partially purified marula peroxidase enzyme. Thermal treatment was carried out at temperatures shown in the graph. Control and residual activity assayed with 2 mM ABTS in citrate–phosphate buffer, pH 4.0, at 25 °C. Values are averages ± SD, $n = 3$.

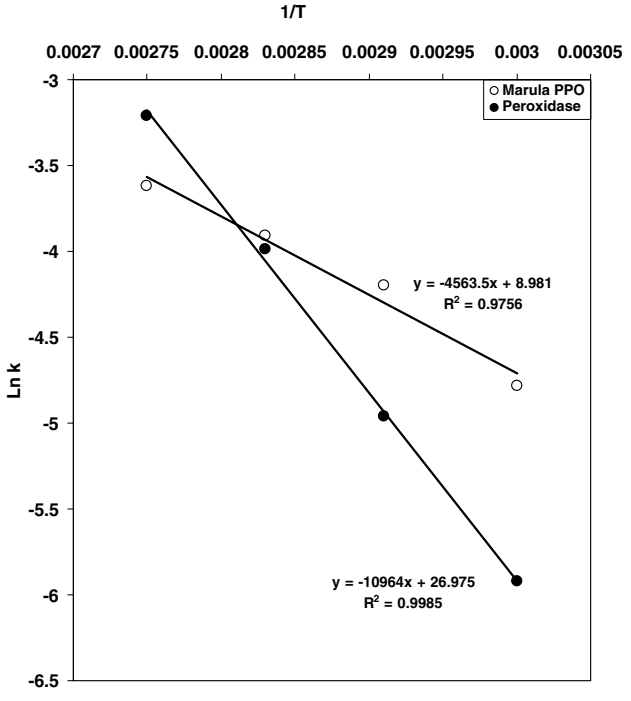

Fig. 12. Arrhenius plot for heat inactivation of partially purified marula polyphenol oxidase and peroxidase.

Some reported values of $\Delta E^{\#}$ for PPO inactivation include 219 kJ mol^{-1} for Dechaunac grape PPO (Lee, Smith, & Pennesi, 1983), 255 kJ mol^{-1} for Ravat grapes PPO, 153 kJ mol^{-1} for Niagara grape PPO (Wissemann & Lee, 1981), 81.2 kJ mol^{-1} for taro PPO (Yemenicioglu, Ozkan, & Cemeroglu, 1999), 109.6 kJ mol^{-1} for Jerusalem artichoke PPO (Zawistowski, Billiaderis, & Murray, 1988), 63 kJ mol^{-1} for medlar PPO (Dincer, Colak, Aydin, Kadioglu, & Guner, 2002), 44 kJ mol^{-1} for persimmon PPO (Ozen et al., 2004) and 18 kJ mol^{-1} for plantain PPO (Ngalani, Signore, & Crouzet, 1993). The $\Delta H^{\#}$ value found in this study is within the range of reported values, albeit at the lower end of the range quoted here. The relatively low value of $\Delta H^{\#}$ for partially purified marula PPO would imply that the marula enzyme is less stable than enzymes quoted above, with the exception of plantain PPO. However, Forsyth et al. (1999) caution against the use of isolated values of $\Delta H^{\#}$ as indicators of enzyme stability.

Reported PPO half life values include 18.8 and 8.5 min at 60 and 70 °C, respectively, for mango kernel PPO (Arogba, Ajiboye, Ugboko, Essitnette, & Afolabi, 1998), 47 and 16 min at 60 and 80 °C, respectively, for Malatya apricot PPO (Arslan, Temur, & Tozlu, 1998), 2 min at 85 °C for mango PPO (Park, Sato, Almeida, & Moretti, 1980), 11.7 and 6.25 min at 70 and 80 °C, respectively, for pear PPO (Halim & Montgomery, 1978), 3.5 and 1.1 min at 65 and 75 °C, respectively, for guava PPO (Augustin, Ghazali, & Hashim, 1985) and 10 min (70 °C) for cabbage PPO (Fujita et al., 1995). Since the half-life values of marula PPO are lower than the values quoted above, it may be concluded that the marula enzyme is less thermostable than the other enzymes. However, it should be borne in mind that the values for marula fruit PPO are derived from the first phase of the bi-phasic thermal inactivation and not the relatively thermostable second phase of heat inactivation.

Some reported peroxidase $\Delta E^{\#}$ values include 81.2 kJ mol^{-1} for taro peroxidase (Yemenicioglu et al., 1999), 67.2 kJ mol^{-1} for HRP type XII (Garcia, Oriega, & Marty, 1998), 148.8 kJ mol^{-1} for Brussels sprout peroxidase (Regalado et al., 1999) and 194 kJ mol^{-1} for okra peroxidase (Yemenicioglu, Ozkan, & Cemeroglu, 1998). The $\Delta H^{\#}$ value of 88.3 kJ mol^{-1} for marula peroxidase is within the range of these values. Some D-values reported for peroxidases in the literature are 8.8 min at 60 °C for Brussels sprout peroxidase (Regalado et al., 1999) and 5.8 min at 60 °C for okra peroxidase (Yemenicioglu et al., 1998). The D-value of 14.5 min for marula fruit peroxidase activity at 60 °C compares favourably with these values.

3.7. Molecular weight determination

The molecular weights of partially purified marula fruit PPO and peroxidase were determined by gel filtration on Sephadex G100. The column was calibrated using a commercial kit (Sigma MW-GF-200 kit) con-

Table 4
Summary of thermal inactivation parameters for partially purified marula PPO and peroxidase

Parameter temperature	$\Delta H^{\#}$ (J mol^{-1})	$\Delta G^{\#}$ (J mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$t1/2$ (min)	D-value (min)
Marula PPO (°C)					
60	38,168	95,117	−180	1.4	4.6
70	35,085	96,381	−179	0.8	2.5
80	35,003	98,424	−180	0.6	1.9
90	34,919	93,472	−161	0.4	1.4
Peroxidase (°C)					
60	88,386	98,266	−29.7	4.3	14.5
70	88,303	98,548	−29.9	1.7	5.5
80	88,220	98,659	−29.6	0.6	2.0
90	88,137	99,184	−30.4	0.3	1.0

taining five proteins of known molecular weight. The resulting calibration graph (not shown) had a linear regression equation of $y = -2.5024x + 14.922$ ($R^2 = 0.9986$). This was used to calculate the molecular weight of marula fruit enzymes. The sample used for molecular weight determination was IEC-fractionated marula enzymes. The molecular weights of PPO and peroxidase were found to be identical – 71,000 Daltons.

Molecular weights comparable to 71 kDa (for IEC-fractionated PPO) include 76 kDa for litchi fruit PPO (Jiang, Fu, Zauberman, & Fuchs, 1999), 72 kDa for Indian tea leaf PPO (Jyotsnabaran, Tamuli, & Bhaduri, 1998) 67 kDa for palmito PPO (Robert, Rouch, Richard-Forget, Pabion, & Cadet, 1996), 70 kDa for plantain PPO (Ngalani et al., 1993) and 69 kDa for potato PPO (Marri, Frazolli, Hochkoeppler, & Poggi, 2003).

According to Robinson (1991) the molecular weight for most plant peroxidases lies within the range 40–50 kDa while Vamos-Vigyazo (1981) indicates a slightly wider range of 30–54 kDa. The molecular weight of 71 kDa for marula peroxidase is outside this range. There are other examples in the literature of plant peroxidases having molecular weight outside the ranges cited here. These examples include 66 and 58 kDa for strawberry peroxidase (Civello, Martinez, Chaves, & Anon, 1995), 60 kDa for peroxidase from black poplar (Tremolieres & Bieth, 1984), 72 kDa for *Solanum bethaultii* trichomes peroxidase (Bouthyette, Eanetta, Hannigan, & Gregory, 1987) and 90 kDa for Brussels sprouts peroxidase (Regalado et al., 1999).

3.8. Isoelectric point determination

Fig. 13 shows the protein elution profiles, enzyme activity profiles and the pH gradients resulting from the chromatofocussing of partially purified marula PPO and peroxidase. The protein elution profile, as determined by the absorbance of the eluate at 280 nm, shows two peaks. The PPO activity profile shows one peak, as does that for peroxidase activity. Significantly, the activity peaks for the two enzymes coincide. The fraction of highest PPO and peroxidase activity coincided with the pH value of 5.43. It was concluded that partially purified marula PPO and peroxidase share the same iso-electric point (pI) of 5.43.

This study describes the isolation and characterisation of PPO and peroxidase from marula fruit. In general, the properties of the individual enzymes are somewhat similar to what is already known about these enzymes. However, the co-elution during IEC and similarity in molecular weight and pI of the two activities from marula fruit raises fundamental questions about, whether the observed phenomena are due to two distinct proteins that simply share similar properties or one protein with two enzymatic

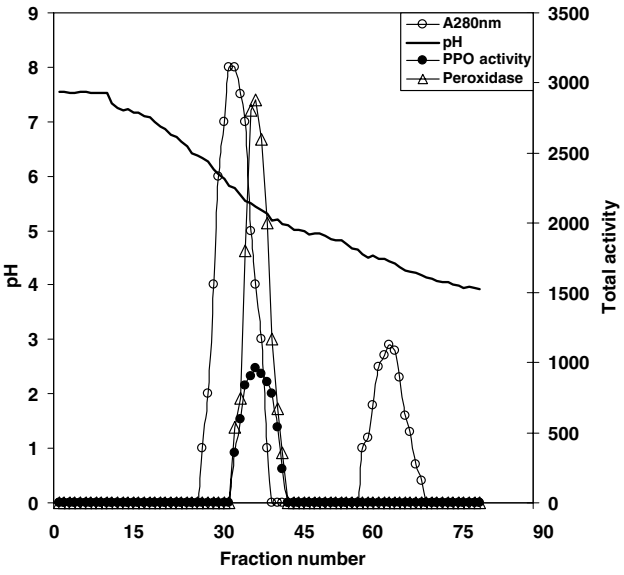

Fig. 13. Chromatofocussing elution profile for marula polyenol oxidase and peroxidase. DEAE fractions with activity were pooled and phosphate buffer (7.0) exchanged with 25 mM imidazole buffer (pH 7.4). Sample was loaded onto PBE 94 column and eluted with Polybuffer 74 (pH 4.0). Aliquots (5 ml) were collected and the pH measured immediately after collection. Fractions were assayed (30 mM catechol) for PPO or peroxidase activity. PPO assay was performed with catechol (30 mM) and MBTH (0.33 mM) in phosphate buffer (100 mM, pH 7.0) and MBTH adduct formation monitored at 500 nm. Peroxidase was determined with ABTS (2 mM) and hydrogen peroxide (0.8 mM) in citrate–phosphate buffer (pH 4.0) and ABTS radical formation was monitored at 414 nm.

activities. This question and the role of peroxidase in browning are currently under investigation

Acknowledgement

We are grateful to Professor Y. Mizrahi (Ben Gurion University of the Negev, Israel) for the generous donation of marula fruit.

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