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Purification and characterization of membrane-bound peroxidases from *Metroxylon sagu*

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Abstract

Two membrane-bound peroxidases, mPOD-I and mPOD-II, have been isolated and purified from *Metroxylon sagu,* using a combination of temperature-induced phase partitioning, DEAE-Toyopearl 650M, CM-Toyopearl 650 M and gel filtration. The mPOD-I and mPOD-II had molecular mass of 51.2 and 43.8 kDa, respectively, as determined by SDS–PAGE. Both enzymes showed high efficiency of interaction with the substrates. The isoenzymes were highly inhibited by ascorbic acid, metabisulfite, L-cysteine and *p*-coumaric acid. The inhibition mode of action and inhibition rate constant (K_i) values for these inhibitors were determined. Their activities were highly enhanced by Al^{3+}, Ca^{2+} and Fe^{3+} but they were moderately inhibited by Zn^{2+}.

Keywords: *Metroxylon sagu*; Pith; Membrane-bound peroxidases; Purification; Characterization

1. Introduction

Sago palm (*Merroxylon sagu*) is considered to be a major source of starch in South East Asia. This plant stores a large amount of starch in its stem, with a higher yield level than that of any other starch crop (Flash, 1997). The starch produced is almost pure, composed of 84–88% carbohydrate (Encyclopaedia Britannica, 1997). Sago palm has potential as a commercial crop, provided good quality starch is available (Ahmad, 1991). The browning of the starch has been one of the most critical problems facing the industry. Phenolic oxidation, mediated by peroxidase, is believed to contribute to deterioration of the colour of the commodity.

Peroxidase (EC 1.11.1.7) from higher plants is an iron-containing enzyme that contains ferriprotoporphyrin III as the prosthetic group (Whitaker, 1994; Wong, 1995). The enzyme is reported to exist in soluble (sPOD) or membrane-bound forms (mPOD) (Robinson, 1991). The membrane-bound peroxidases (mPOD) are often of a cationic type that can be adsorbed on a cation-exchange chromatographic material (Billaud, Louarme, & Nicolas, 1999; Boeuf, Bauw, & Rambour, 2000; Civello, Martinez, Chaves, & Anon, 1995; Forsyth & Robinson, 1998; Lee & Kim, 1994; Lopez-Serrano & Barcelo, 1996; Moulding, Goodfellow, Mclellan, & Robinson, 1989). Multiple ranges of isoenzymes, differing with respect to molecular mass, thermal stability, pH optimum, substrate specificity and physiological role have been reported (Robinson, 1991). Two mPOD isoenzymes were isolated from Korean radish with molecular masses of 45.0 and 44.0 kDa (Lee & Kim, 1994) and those from strawberry were 58.1 and 65.5 kDa (Civello et al., 1995). However, mPOD from wheat germ (Converso & Fernandez, 1995) and potato (Boucoiran, Kijne, & Recourt, 2000) showed no isoenzymes.

Plant peroxidases have specific requirements for peroxide and may be considered as peroxide scavengers (Robinson, 1991). In the presence of peroxide, the peroxidases from plant tissues were able to oxidize a wide range of phenolic compounds, such as guaiacol, pyrogallol, chlorogenic acid, catechin and catechol (Richard-Forget & Gauillard, 1997; Vamos-Vigyazo, 1981; Whitaker, 1994). Oxidation of a wide range of phenolic compounds, has led to speculation that they may be associated with losses in colour and flavour of raw and processed foods (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994).

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In the course of the study of the involvement of latent polyphenol oxidase in enzymatic browning reactions in *M. sagu* (Onsa, Saari, Jinap, & Bakar, 2000), we also found strong peroxidase activity. However, little is known about POD in *M. sagu* particularly membrane-bound peroxidase. In order to develop a method for preventing the enzymatic browning reaction, a more precise understanding of the implication of mPOD in enzymatic browning reaction of *M. sagu* is required. This study was initiated to purify mPOD from *M. sagu* using a temperature-induced phase partitioning technique in combination with a conventional purification procedure and the properties were determined.

2. Materials and methods

2.1. Plant material

Sago (*M. sagu*) pith was collected from Batu Pahat, Johor, Malaysia. Five kilograms of sago pith were obtained from a 1–2 year-old tree with a trunk height of 2.8 m and a diameter of 0.5 m. A log of 0.2 m height was selected from a trunk 1 m from the crown and 1 m above the base. The log was debarked and divided into four equal parts and stored at 4 °C until used within 4–6 days.

2.2. Reagents

Triton X-114 and Tris-base were obtained from Sigma Chemicals. Guaiacol was purchased from Wako Pure Chemical Industry, Japan. Sodium acetate, mono and disodium phosphates were obtained from Fluka. DEAE-Toyopearl 650M and CM-Toyopearl 650 M were obtained from TOSOH Corporation, Japan.

2.3. Enzyme isolation

The log was divided into four equal parts and two parts were selected randomly. A sample of 5.0 kg of sago pith was cut into small pieces using a stainless steel knife. The enzyme was extracted from the cell membrane fractions of fresh sago log, as described by Onsa et al. (2000).

2.4. Enzyme purification

2.4.1. Temperature-induced phase partitioning

Membrane-bound peroxidase (mPOD) extract was further subjected to temperature-induced phase partitioning, as described by Onsa et al. (2000). The clear supematant was considered to be a partially-purified enzyme and was used for further purification.

2.4.2. Anionic chromatography

The partially-purified enzyme was applied to a DEAE–Toyopearl 650 M anion-exchange column chromatograph (2.5×15 cm), pre-equilibrated with 0.01 M Tris buffer, pH 7.8. The enzyme solution (84 ml) applied was eluted with a linear gradient, from 0 to 0.2 M NaCl in 0.01 M Tris buffer, pH 7.8. Elution was carried out at a flow rate of 30 ml h^{-1} and fractions of 5 ml $tube^{-1}$ were collected using a fraction collector (Bio Rad, USA). Each fraction was assayed for mPOD activity. The amount of protein in each fraction was estimated by measuring the absorbency at 280 nm in a UV–visible spectrophotometer, using Shimadzu UV-1601, (Shimadzu Co, Japan).

2.4.3. Cationic chromatography

The fractions with POD activity that were not adsorbed on the DEAE-column were pooled, dialysed against 0.01 M acetate buffer, pH 4.7, and applied to CM-Toyopearl 650 M (2.5×15 cm). The column was pre-equilibrated with the same acetate buffer, pH 4.7. Elution was done by a linear gradient of the same buffer from 0 to 0.2 M NaCl at a flow rate of 30 ml h^{-1}. Fractions of 5 ml $tube^{-1}$ were collected and assayed for mPOD activity and protein content.

2.4.4. Gel filtration

Fractions with mPPO activity, eluted by CM-Toyopearl 650 M chromatography, were dialysed against three changes of 0.01 M Tris buffer, pH 6.8, overnight. The fractions were then concentrated by ultrafiltration (Advantec UHP-43), using membrane filter PM 10 (Amicon Co.). The concentrates were cleared by centrifugation at 20,000*g* for 10 min using a refrigerated centrifuge (Kubota, 7800). The supernatant was applied to Sephadex G-100, pre-equilibrated with 0.1 M Tris–HCl, pH 6.8, and eluted with the same buffer at a flow rate of 15 ml h^{-1}. The fractions collected were 3 ml $tube^{-1}$.

2.5. Peroxidase activity assay

POD activity was determined according to the method described by Fujita et al. (1995) with some modifications. The reaction mixture contained 1.8 ml of 0.1 M acetate buffer, pH 4.5, 1.0 ml of 0.03 M guaiacol as a substrate, 0.1 ml H_2O_2 and 0.1 ml of enzyme solution. The activity was assayed by recording the increase in optical density at 420 nm for 3 min at 20 s intervals at 25 °C using a spectrophotometer (Shimadzu UV-1601) equipped with an enzyme kinetics software package. The reaction rate was calculated from the initial slope of the progress curve. One unit of enzyme activity was defined as the amount of enzyme causing a 0.001 change in the absorbency per min.

2.6. Determination of protein concentration

Protein concentration was determined by the Hartree method (1972), using bovine serum albumin as standard protein.

2.7. Determination of molecular masses

The molecular masses of sago pith mPOD isoenzymes were determined by SDS–PAGE, as described by Onsa et al. (2000).

2.8. Effect of pH

Enzyme activity was determined with 0.03 M guaiacol, in a range of pH 3–9 (sodium acetate buffer, 3.0–5.5; citrate–phosphate buffer, 5.5–7.0; Tris-buffer, 7.0–8.0 and glycine buffer, 8.0–9.0). Assay was performed at 25 °C in duplicate and the averages of duplicate readings and at the point of pH overlaps were taken. The ionisation rate constant was determined by plotting the log of enzyme activity versus the pH, following the procedure of Dixon & Webb (1979) and fitted to the equation below:

$$\text{Log } V_{\text{m}}^{\text{H}^+} = \text{Log } V_{\text{m}} - \text{Log}\left(1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+]\right) \quad (1)$$

where, $V_{\text{m}}^{\text{H}^+}$ is the maximum velocity at a given pH and H^+ is the hydrogen ion concentration. The constants K_1 and K_2 represent the dissociation constant of the enzyme functional groups reflected at the acid and basic side of the pH profile, respectively.

2.9. Determination of K_m and V_m

Specificity was determined for the mPOD isoenzymes from *M. sagu* by determining the reaction rate constant (K_{m}) and the maximum velocity (V_{m}) for selected monophenols (guaiacol and *p*-cresol), diphenols (chlorogenic acid, catechin, epicatechin, catechol and 4-methylcatechol), triphenol (pyrogallol) and TMBZ in a concentration range of 0.01–0.1 M. The reaction of each substrate was measured at the absorption maximum of the corresponding quinone products, following the procedure suggested by Zhou, Smith, & Lee (1993). The data were plotted according to Lineweaver and Burke (1934). The relative maximum activities were calculated by considering activity with guaiacol as 100%.

2.10. Effect of inhibitors

The reaction mixture contained 0.1 ml enzyme solution, 0.9 ml acetate buffer, pH 5.0, and 1.0 ml of inhibitor solution at varying concentrations. Inhibitors tested were halides (400 mM NaCl and KI), antioxidants (0.05 mM ascorbic acid and sodium metabisulfite, and 3.0 mM kojic acid), carboxylic acids (20 mM *p*-coumaric acid and benzoic acid), metal chelator (3.0 mM EDTA) and thiol compounds (0.2 mM L-cysteine and 1.0 mM thiourea). The reaction was initiated by addition of 1.0 ml of 0.03 M guaiacol and 0.1% H_2O_2 as mPOD substrate. The percentage inhibition was expressed as relative to enzyme activity without inhibitor. A lag phase was observed in the presence of certain inhibitors. On occurrence of a lag phase, the activity was calculated from the rate of change in absorbency after the lag phase, as suggested by Valero, Varon, and Garcia-Carmona (1992).

2.11. Determination of inhibition kinetic analysis

The inhibition kinetic analysis of *M. sagu* mPOD was determined for the most effective and food compatible inhibitors of mPOD isoenzymes The reaction was carried out with guaiacol in a concentration range of 0.01–0.1 M and 0.1% H_2O_2 without inhibitor and in the presence of inhibitors at three different concentrations, namely *p*-coumaric acid (15.0, 10.0 and 5.0 mM), kojic acid (3.0, 1.5 and 0.5 mM), ascorbic acid (0.04, 0.02 and 0.01 mM), sodium metabisulfite (0.04, 0.02 and 0.01 mM), and L-cysteine (0.15, 0.1 and 0.05 mM). The inhibition mode of action was determined from the Lineweaver–Burk plot of $1/V_0$ activity against 1/[S]. The K_{i} value was determined from the slope of Lineweaver–Burk plot against the concentration of corresponding inhibitor, as described by Segel (1976).

2.12. Effect of metal ions

The effects of various metal ions were determined by pre-incubating each isoenzyme with the individual ions in 0.1 M acetate buffer at pH 5.0 for 10 min. The activity of the enzyme was then determined, under standard assay conditions, in the presence and absence of metal ions.

3. Results and discussion

3.1. Isolation of mPOD

The mPODs were successfully isolated from *M. sagu* cellular fractions with Triton X-114. The extract had a specific activity of 8×10^2 units mg^{-1} protein when tested with 0.03 M guaiacol in the presence of 0.1% H_2O_2 (Table 1). The mPOD formed a mix micelle with Triton X-114 and thus can be characterized as having a non-polar residue forming a hydrophobic exterior that makes a micelle-like structure with the detergent (Bordier, 1981). High amounts of activity, up to 85.7%, were retained after the extract had been osmotically shocked

Table 1
Purification of mPOD isoenzymes (mPOD-I and mPOD-II) from *Metroxylon sagu*

Steps	Volume (ml)	Total protein (mg)	Total activity (unit$\times 10^3$)	Specific activity (units mg^{-1} protein)$\times 10^3$	Yield (%)	Purification factor (fold)
Crude extract	170	734	598	0.81	100	1
Supernatant of 1.5% TX-114 extract	150	353	513	1.46	85.7	1.80
Supernatant of 8% TX-114 extract	120	90.0	420	4.67	70.2	5.83
DEAE Toyopearl-650 M						
Flow through fractions	110	52.5	384	16.6	64.1	20.5
CM-Toyopearl 650 M						
mPOD-I	42	1.68	99.1	59.0	16.6	72.8
mPOD-II	90	7.68	157	21.8	26.2	26.9
Sephadex G-100						
rnPOD-I	25	0.75	46.5	62.0	7.8	76.5
mPOD-II	30	1.8	34.5	30.0	5.8	37.0

using high-speed centrifugation, with enhancement in the specific activity of the crude extract (1.8-fold). The extract was turbid and brownish, due to the presence of large amounts of phenols and other impurities that were then separated by a temperature-induced phase partitioning technique. This technique was used for the first time to isolate mPOD from plant tissues and resulted in a 5.8-fold purification of *M. sagu* mPOD. Rodriquez-Lopez et al. (2000) employed a temperature-induced phase partitioning technique to isolate soluble POD from melon fruit. In other experiments, 0.5% Triton X-100 was reported to considerably enhance the isolation of POD activity from blackberry fruits (Gonzalez, Ancos, & Cano, 2000), but it did not affect the extraction of mPOD from sweet potato tuber (Leon et al., in press).

3.2. Purification of mPOD

The partially purified enzyme was further subjected to DEAE-Toyopearl 650 M chromatography. Fig. 1 shows the elution profile of mPOD after DEAE-Toyopearl 650 M chromatography. The first bulk of protein eluted at the washing phase by 0.01 M Tris–HCl pH 7.8 showed a high mPOD activity when assayed with 0.03 M guaiacol and 0.1% H_2O_2. It was observed that the protein with no mPOD activity was eluted at a slightly faster rate than that of the mPOD activity. High mPOD activity in the flow-through fractions explains that positive charges predominate at the surface structure of the enzyme, which prohibits electrostatic interaction with the anion-exchanger. Although no adsorption occurred on the DEAE-Toyopearl 650 M, the specific activity of the flow-through fraction of mPOD was greatly enhanced (by 20.5-fold, Table 1).

The flow-through fractions (Nos. 36–57) from DEAE-Toyopearl with mPOD activity were pooled and dialyzed against 0.01 M acetate buffer, pH 4.7, and applied to a CM-Toyopearl 650 M column (1.5×15 cm). The enzyme was highly adsorbed on the column and could only be eluted with a linear gradient of 0–0.2 M NaCl in 0.01 acetate buffer, pH 4.7. At pH 4.7, the acetate buffer, with Na^+ as counter ions used for equilibration of the CM-Toyopearl 650 M, has a high buffering power (0.5 mM), which allows biospecific adsorption of mPOD to the cationic material (Scopes, 1993).

The elution profile showed two protein peaks with high mPOD activity. The first bulk of bound protein with high mPOD activity was eluted at ~0.09–0.10 M NaCl in acetate buffer, pH 4.7, and designated as mPOD-I (Fig. 2). The second peak, eluted with 0.11–0.13 M NaCl, was designated as mPOD-II. At this stage mPOD-I and mPOD-II were purified by 72.8- and 26.9-fold, respectively (Table 1). This elution profile suggested that these two mPOD isoenzymes are cationic enzymes that possess positive net surface charges, which allow them to be adsorbed onto a negatively charged surface, such as CM-Toyopearl 650 M. Similar elution characteristics were reported for mPOD from chicory cell suspension when loaded onto CM-cellulose equilibrated with 0.01 M acetate buffer, pH 5.0; the first peak eluted at 0.25 M NaCl and the second peak at 0.35 M NaCl (Boeuf et al., 2000). Similar observations were also reported for mPOD from *Vaccinium myritillus* (blueberry) which possesses two isoenzymes binding to CM-sepharose and eluted at ~0.08 and ~0.05 M NaCl. Ionically-bound peroxidases are often reported to be cationic enzymes; this property allowed the enzyme to be adsorbed to a cationic exchange material (Moulding et al., 1989). High adsorption of plant mPOD onto the cationic exchange material has been reported for strawberry fruit eluted from CM-cellulose at ~0.6 M NaCl when loaded on 0.2 M acetate buffer, pH 4.8 (Civello et al., 1995), while, mPOD from strawberry fruit is highly adsorbed on CM-cellulose and eluted only at 1.0 M NaCl (Lopez-Serrano & Barcelo, 1996).

Each isoenzyme was finally concentrated using ultrafiltration (PM 10), and applied separately onto a Sephadex G-100 column and eluted with 0.1 M Tris–HCl

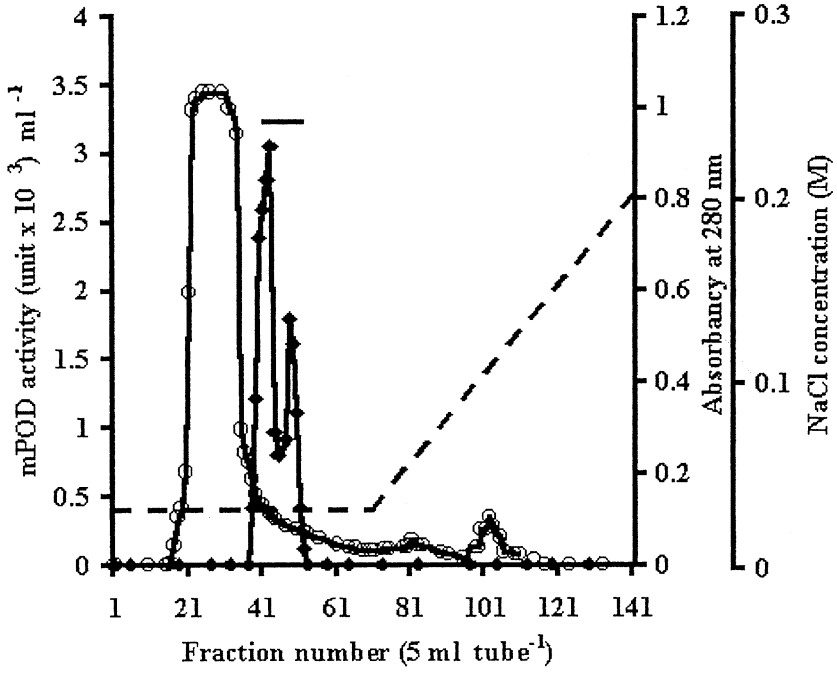

Fig. 1. Elution profile of the DEAE-Toyopearl 650 M chromatography of mPOD temperature-induced phase partitioning extract from *Metroxylon sagu*. (◆) mPOD activity (unit×10^3) ml^{-1}, (○) absorbancy at 280 nm, (. . .) gradient elution with NaCl (0–0.2 M).

buffer, pH 6.8. The procedure enhanced the purification of mPOD-I and mPOD-II by 76.5- and 37.0-fold, and 7.8 and 5.8% of the activities were resolved, respectively. The specific activities of 62.0×10^3 and 30.0×10^3 units mg^{-1} protein for mPOD-I and mPOD-II, respectively were higher than the mPOD from chicory (80.0 units mg^{-1} protein; Boeuf et al., 2000) or strawberry (116 units mg^{-1} protein; Civello et al., 1995). Fractions with mPOD-I and mPOD-II activity, recovered from gel filtration, were used for characterization and kinetic study.

3.3. Molecular masses

mPOD-I and mPOD-II from *M. sagu* are monomeric, as indicated by the presence of two single protein bands (Fig. 3). Their molecular masses were 51.2 and 43.8 kDa, respectively (Fig. 4). These isoenzymes could possibly be genetically independent units and they differ in their primary structures (Beltiz & Grosch, 1999; Robinson, 1991). The existence of multiple isoenzymes of mPOD with different molecular masses has been reported for *Vaccinium* spp. (34.0 and 38.0 kDa; Melo, Cabral, & Fevereiro, 1995) and Brussels sprouts (32.0 and 26.0 kDa; Forsyth & Robinson, 1998) However, two isoenzymes with identical molecular masses were reported for barley grain mPPO at 37 kDa (Rasmussen et al., 1997) and sweet potato, sPOD, at 39.5 kDa (Lin, Chen, & Zhang, 1996), when using analytical gel electrophoresis. The molecular masses of *M. sagu* mPOD isoenzymes are higher than those reported for mPOD from wheat germ, with a molecular mass of 35.0 kDa (Converso & Fernandez, 1995), and potato tuber sprout at 38.0 kDa (Boucoiran et al., 2000). The molecular mass of mPOD-II is similar to that of Korean radish at 45.0 and 44.0 kDa (Lee & Kim, 1994). Other mPOD showed higher molecular masses, e.g. strawberry mPOD isoenzymes (58.1 and 65.5 kDa; Civello et al., 1995) and seeds of *Araucaria araucana* (83.0 kDa; Riquelme & Cardemil, 1993). These reports attribute the higher molecular masses to a glycosylated protein.

3.4. Effect of pH

pH is a determining factor in the expression of enzymatic activity as it alters the ionization states of amino acid side chains or the ionization of the substrate (Voet and Voet, 1990). The effect of pH on the activity of *M. sagu* mPOD isoenzymes is shown in Fig. 5. The isoenzymes, mPOD-I and mPOD-II, showed maximum activities at pH 6.0 and 5.5, respectively. When sago starch was extracted and washed with water, the pH of the slurry was near to 5.5. Thus, in this condition, the catalytic expression of both mPOD isoenzymes is favoured and can highly contribute to the browning reaction. The result obtained here is similar to that reported for mPOD isoenzymes from Korean radish, at pH 5.0 and 6.5 (Lee & Kim, 1994), wheat germ at pH 5.3 and 6.3 (Billaud et al., 1999) and *Opuntia* at pH 5.7 (Padiglia, Pazzaglia, Cruciani, Medda, & Floris, 1995), but slightly higher than mPOD from potato tuber sprouts and potato tuber (4.0 and 4.5, respectively) (Boucoiran et al., 2000). However, Lopez and Burgos

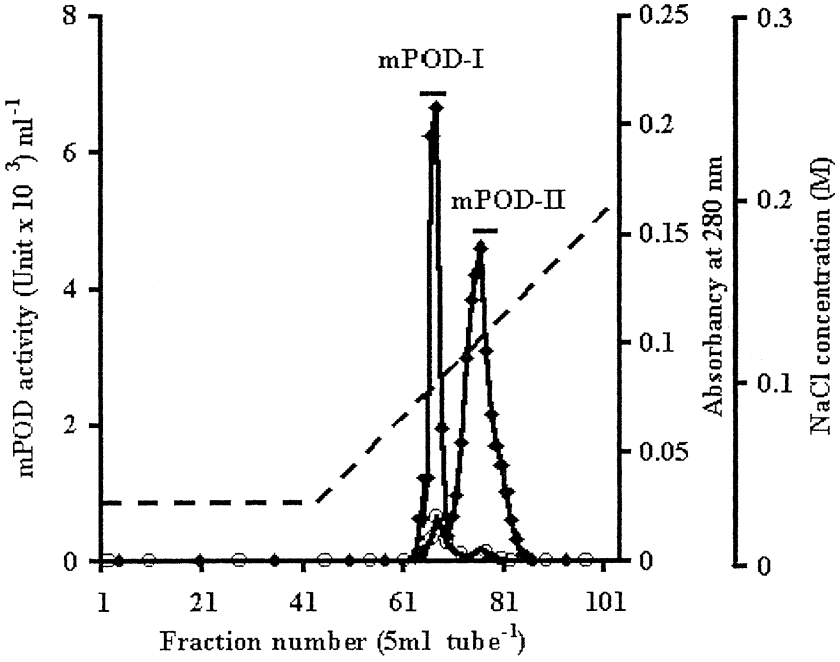

Fig. 2. Elution profile of the CM-Toyopearl 650 M chromatography *of* the flow through fraction from DEAE-Toyopearl 650 M. (◆) *m*POD activity (unit×10^3) ml^{-1}, (○) absorbency at 280 nm, (. . .) gradient elution with NaCl (0–0.2 M). Fractions are combined as indicated by the bars.

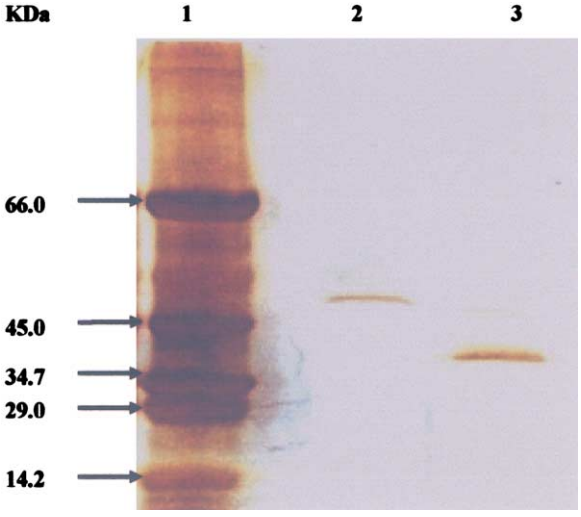

Fig. 3. SDS–PAGE of membrane-bound peroxidases from *Metroxylon sagu:* Lane 1, Low molecular mass protein markers (14.2–66.0 kDa), lane 2, mPOD-I (2.2 μg), and lane 3, mPOD-II (2.4 μg). mPOD isoenzymes recovered from Sephadex G-l00 were loaded onto 10% SDS–PAGE and the gel was silver stained.

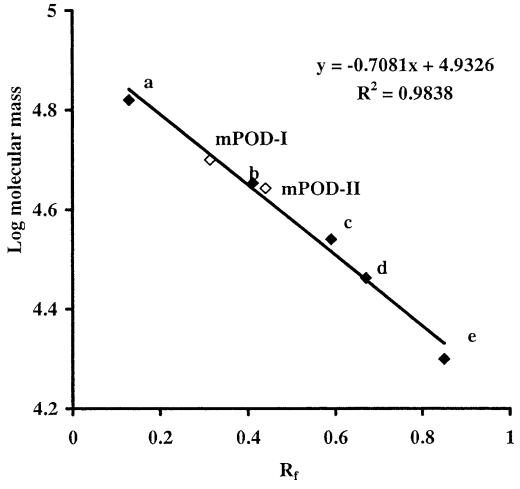

Fig. 4. Molecular masses *of* the *Metroxylon sagu* mPOD isoenzymes. (◇) The protein markers used [a]bovine albumin (66.0 kDa), [b]pepsin (34.7 kDa), [c]carbonic anhydrase (29.0 kDa), [d]trypsinogen (24.0 kDa), and [e]lactoglubulin (14.2 kDa), (◆) mPOD isoenzymes molecular masses. The molecular mass was determined using SDS–PAGE on 10% gel electrophoresis.

(1995) reported that the release of the heme group from the enzyme active site was pH dependent and occurred most rapidly below pH 5 and led to loss of POD activity.

The active site on enzymes is frequently composed of ionized groups (prototropic groups) that must be in the proper ionic form in order to maintain the conformation of the active site, enzyme-substrate binding, or reaction catalysis (Whitaker, 1994). The behaviour of the enzyme activity at different pH values can provide information concerning the identities of the prototropic groups at the active site once the pK values are known (Segel, 1994; Whitaker, 1994). The pK values of the essential ionizing groups involved in the catalytic process were determined for mPOD-I and mPOD-II isoenzymes, following the Dixon–Webb plot (1979). As presented in Fig. 6a,b, the pH profile of mPOD-I showed decreases in Log V_m at low pH, with positive slope, and at high pH, with negative slope. From Eq. (1), mPOD-I shows pK_1 and pK_2 values of 4.2 and 7.4,

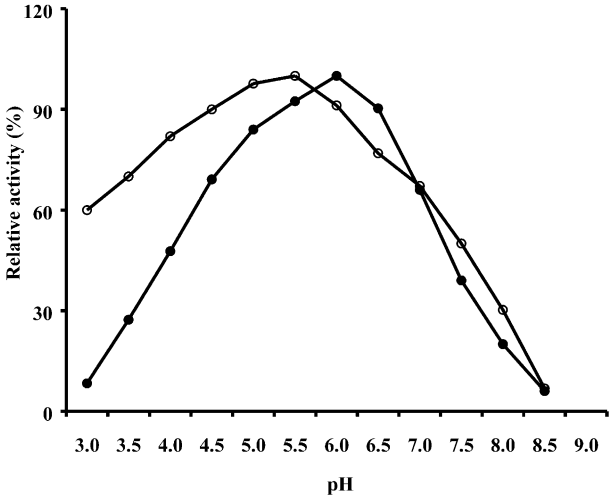

Fig. 5. . Effect of pH on the activity of membrane-bound peroxidases from *Metroxylon sagu*. The activity assayed with 0.03 M guaiacol and 0.1% H_2O_2 as a substrate and 0.1 M buffer (sodium acetate buffer pH 3.0–5.5, citrate–phosphate buffer pH 5.5–7.0, Tris-buffer pH 7.0–8.0, and glycine buffer pH 8.0–9.0). (•) mPOD-I (○) mPOD-II.

respectively. Whitaker (1994) suggests that aspartate and cysteine could possibly be involved in the catalysis step of mPOD-I.

M. sagu mPOD-II has one ionizable group involved in the catalysis process, with a pK_2 value of 7.5. This neutral or alkaline group could possibly be due to histidine (Whitaker, 1994). The ionization rate constant pK ~4.0 was reported for horseradish mPOD (Kato, Aibara, Morita, Nakatani, & Hiromi, 1984). However it is difficult to assign an experimental pK value to the reactive group of an amino acid because it is based on approximation. These hypotheses could be confirmed by specific chemical modifications of the active site.

3.5. K_m and V_m values of mPOD isoenzymes

M. sagu mPOD isoezymes hardly show any activity toward phenols in the absence of H_2O_2. They show a tendency to accept a wide range of hydrogen donors, including polyphenols, only in the presence of H_2O_2. Their K_m and V_m values are presented in Table 2. Using guaiacol as a reducing substrate, the activity of mPOD isoenzymes showed a Michaelis–Menten relationship at varying H_2O_2 concentrations. The K_m values of H_2O_2, calculated from the Lineweaver–Burk plot, are 0.08 and 0.06 mM for mPOD-I and mPOD-II, respectively. The significance of low K_m values for H_2O_2 reflect a high number of H_2 or hydrophobic interactions between the substrate and the heme group at the enzyme active site (Richard-Forget & Gauillard, 1997). This affinity of interaction with H_2O_2 was higher than that reported for mPOD from other plants, e.g. mPOD from pear (1.5 mM; Richard-Forget & Gauillard, 1997), chicory cell

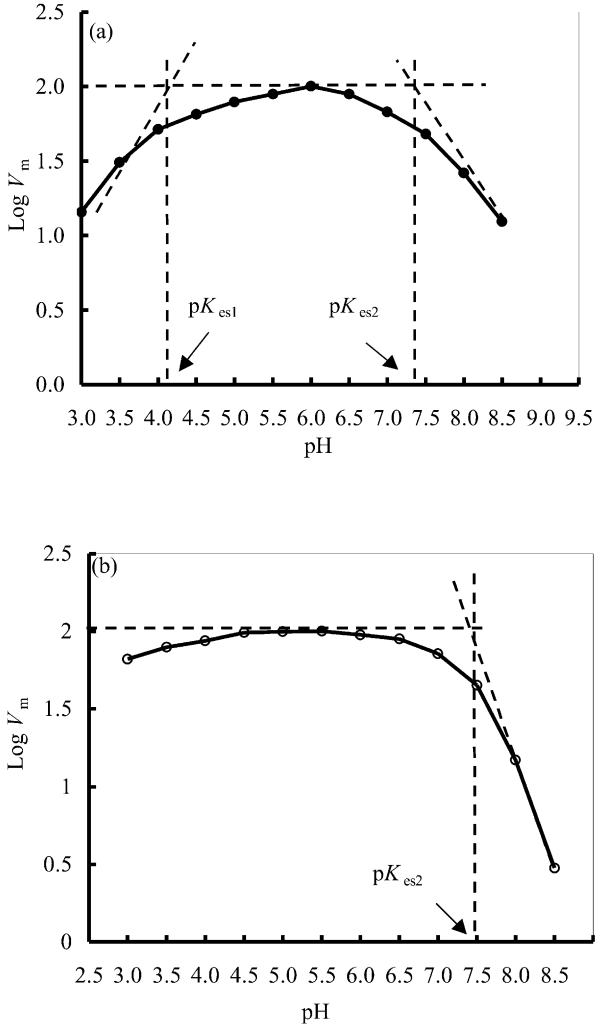

Fig. 6. Dixon–Webb plot for determination of pK_1, and pK_2 values for mPOD isoenzymes from *Metroxylon sagu*. Log V versus pH with 0.03 M guaiacol and 0.1% H_2O_2 as a substrate: (a) mPOD-I and (b) mPOD-II.

suspension culture (2.4 mM; Boeuf et al., 2000), Korean radish (0.19 and 0.72 mM; Lee & Kim, 1994), Brussels sprouts (11.4 and 6.2 mM; Regalado, Arvizu, & Garcia-Almendarez, 1999) and *Araucaria* seeds (13.6 mM; Riquelme & Cardemil, 1993). However, Duarte-Vazquez, Garcia-Almendarez, Regalado, and Whitaker (2000) reported lower affinity for H_2O_2 interaction with turnip mPOD isoenzyme Cl at 0.04 mM, while the isoenzymes C2 and C3 showed higher K_m values of 0.25 and 0.85 mM.

Guaiacol is the most commonly used substrate for peroxidase activity assay (Adams, Harvey, & Dempsey, 1996; Gillikin & Graham, 1991; Soda, Hasegawa, Suzuki, & Ogura, 1991). Although mPOD isoenzymes from *M. sagu* showed a high V_m for guaiacol, their ability to oxidize this substrate is low, due to their low affinity for the enzyme, with high K_m values of 32.2 and 22.9 mM for mPOD-I and mPOD-II, respectively

(Table 2). However, in terms of catalytic efficiency (V_m/K_m), mPOD-I was found to follow the order: chlorogenic acid > epicatechin > catechin > guaiacol > pyrogallol > 4-methylcatechol > catechol. With mPOD-II the efficiencies for different phenols showed a slightly different order: chlorogenic acid > pyrogallol > epicatechin > guaiacol > catechin > 4-methylcatechol > catechol. High efficiencies of chlorogenic acid, catechin and epicatechin oxidations by mPOD were due to their high affinities for enzyme, as indicated by the low K_m values of 1.6, 5.2 and 5.2 mM for mPOD-I, and 2.1, 3.0 and 3.5 for mPOD-II. This trend is similar to some other plant POD enzymes, as reported for sPOD from pear (Richard-Forget & Gauillard, 1997) and royal palm leaves (Sakharov, Vesga, Galaev, Sakharova, & Pletushkina, 2001). High interactions with chlorogenic acid, catechin and epicatechin by mPOD isoenzymes proved the effective involvement of the enzyme in enzymatic browning of *M. sagu*.

It should be emphasized that *M. sagu* mPOD isoenzymes were highly reactive towards the aromatic amine; tetramethylbenzidine (TMBZ) that was oxidized 60- and 100-fold faster than guaiacol. The efficiency of TMBZ is unusually higher than the values calculated for the phenolic compounds tested. mPO-II had a higher efficiency for TMBZ, with V_m/K_m at 237 compared with mPOD-I at 134. TMBZ could be considered as the best substrate for both mPOD isoenzymes but it is not recommended for routine analysis because of its low solubility and carcinogenic effect (Agostini, Medina, Milrad, & Tiger, 1997).

It is observed that the two mPOD isoenzymes displayed different affinities of interaction with different phenols containing 1, 2 or 3 hydroxy groups. The variation in affinity was irrespective of the number of OH groups present in the phenol. At present there is no obvious explanation at the molecular level for different reactivities of mPOD isoenzymes towards different substrates. However, according to Banci (1997) the electrostatic field produced by the charged residues of the enzyme would affect the reduction potential of the active site.

Table 2
K_m and relative V_m parameters for mPOD-I and mPOD-II from *Metroxylan sagu*

Substrates	mPOD-I			mPOD-II		
	K_m (mM)	V_m(%)[a]	V_m/K_m	K_m (mM)	V_m(%)	V_m/K_m
Monophenols						
Guaiacol	32.2	100	3.1	22.9	100	4.4
p-Cresols	–	–	–	–	–	–
Diphenols						
Chlorogenic acid	1.6	121	75.6	2.1	206	98.2
Catechin	5.2	17.9	3.4	3.0	10.7	3.6
Epicatechin	5.2	28.4	5.5	3.5	16.4	4.7
Catechol	25.0	26.6	1.1	20.0	22.0	1.1
4-Methylcatechol	22.0	30.8	1.4	17.5	45.0	2.5
Triphenols						
Pyrogallol	32.2	101	2.1	14.2	162	11.4
TMBZ	7.2	966	134	2.6	6167	237
Peroxide[b]	0.08	125	1562	0.06	146	2433

[a] V_m% is Max velocity as percent of that of guaiacol.
[b] 0.03 M Guaiacol used as a reducing substrate.

3.6. Effect of inhibitors

At present, the inhibition of POD is rarely reported, because the focus of published research is a potential substitute for commercial horseradish peroxidase (Duarte-Vazquez et al., 2000). In this study, complete inhibition of *M. sagu* mPODs was achieved by using 0.05 mM sodium metabisulfide and 0.05 mM ascorbic acid (Table 3). mPOD-I was also completely inhibited in the presence of 20 mM *p*-coumaric acid but this inhibited mPOD-II to only 96%. Both mPOD isoenzymes were strongly inhibited in the presence of 3.0 mM kojic acid and 0.2 mM L-cysteine (87–92%). On the other band, moderate inhibition was observed in 1.0 mM thiourea, which inhibited mPOD-I and mPOD-II by 79 and 73%, respectively. Poor inhibitory effect was observed for the halide ions, 400 mM NaCl (60–44%) and 400 mM KI (35–30%). EDTA inhibited both isoenzymes moderately (60–75%). EDTA, as a metal chelator, was unable to fully combine with Fe^{3+} ions; consequently, the active site maintained its integrity. Fujita et al. (1995) and Lin et al. (1996) also reported

Table 3
Effects of different inhibitors on the activity of mPOD isoenzymes from *Metroxylon sagu*

Type of inhibitor	mPOD isoenzymes		
	[Inhibitor] (mM)	mPOD-I (% inhibition)	mPOD-II (reactivity)
(1) Halides			
NaCl	400	60	44
KI	400	35	30
(2) Metal chelator			
EDTA	3.0	60	75
(3) Antioxidants			
Ascorbic acid	0.05	100	100
Metabisulfite	0.05	100	100
Kojic acid	3.0	87	88
(4) Carboxylic acid			
p-Coumaric acid	20.0	100	96
Benzoic acid	20.0	40	25
(5) Thiol compounds			
L-Cysteine	0.2	92	90
Thiourea	1.0	79	73

unsuccessful inhibition of POD by EDTA and 20 mM benzoic acid.

3.7. Inhibition rate constant of selected inhibitors

The inhibition kinetics were determined for the most effective and food-compatible inhibitors, following the procedure of Lineweaver–Burk (1934). At present no report has been found on inhibition kinetics on POD, and thus the information presented in this study adds to the understanding of the physical and chemical nature of the enzyme. Table 4 shows the inhibitors tested with *M. sagu* mPOD. *p*-Coumaric acid and ascorbic acid were found to be competitive inhibitors, while both kojic acid and sodium metabisulfite were non-competitive. Ascorbic acid was the most potent inhibitor with K_i value of 0.01 mM, followed by sodium metabisulfite, L-cysteine, kojic acid and *p*-coumaric acid.

Competitive inhibition was observed for *p*-coumaric acid with guaiacol and H_2O_2 as a substrate (Fig. 7a) with K_i value of 1.97 mM. This mode of inhibition explains that *p*-coumaric acid binds only to the active site of the free enzyme, prohibiting the substrates from undergoing oxidation. *p*-Coumaric was considered to be a substrate analogue for plant POD and is reported to be the best substrate for some other POD enzymes such as mPOD from *Aloe barbadensis* (Esteban-Carrasco, Zapata, Lopez-Serrano, Sabater, & Martin, 2000)

Inhibition by kojic acid is purely non-competitive, as illustrated in Fig. 7b. Kojic acid is known as a reducing agent that has a tendency to interact with the heme iron at the active site. This result suggests that kojic acid did not react with the free enzyme but reduced the quinone component back to diphenol as occurred for some other PPO enzymes (Chen, Wei, & Marshall, 1991). The affinity of inhibition of *M. sagu* mPOD by kojic acid was determined, by the K_i value, to be 0.44 mM.

Ascorbic acid and sodium metabisulfite are most often used in industrial practice to prevent browning of processed fruits and vegetables (Vamos-Vigyazo, 1981). The action of ascorbic on mPOD from *M. sagu* is by competitive inhibition (Fig. 7c), with highest potency, i.e. a K_i value of 0.01 mM. However, the result was different from that reported for mPOD isolated from potato tuber mPOD which was highly reactive with ascorbic acid in the presence of H_2O_2 (Leonardis, Dipierro, & Dipierro, 2000). From Fig. 7d, the inhibition of mPOD by sodium metabsulfite is a purely non-competitive action. Non-competitive inhibition is observed when the inhibitor combines with the enzyme substrate complex and prevents transformation of the substrate to product; the substrate and the inhibitor do not compete with each other in the binding to the enzyme active site (Segel, 1994; Wong, 1995). It has been suggested that the metabisulfite is involved in reducing the H_2O_2; by eliminating the H_2O_2 it blocks enzyme activity and maintains the donor substrate in its reduced form (Vamos-Vigyazo, 1981). The metabisulfite showed a high potency for inhibiting mPOD, with a K_i value of 0.024 mM.

On the other hand, L-cysteine exhibited uncompetitive inhibition with *M. sagu* mPOD (Fig. 7e). Uncompetitive inhibition occurs when the inhibitor binds to the enzyme substrate complex (Palmer, 1995; Segel, 1994). This result reveals that L-cysteine has no direct effect on the free enzyme but it binds the quinone intermediates, thus preventing the formation of the browning pigment (Segel, 1994). L-cysteine was able to a form quinone coupler, which is a stable colourless compound. The possibility of reduction of the quinone produced by enzyme catalytic activity using L-cysteine has also been reported (Friedman & Bautista, 1995). L-cysteine was found to be a potent inhibitor of mPOD (after ascorbic acid and sodium bisulfite) with a K_i value of 0.03 mM.

Table 4
Inhibition modes and inhibition constants (K_i) of selected inhibitors for purified mPOD from *Metroxylon sagu*

Inhibitors	Inhibition mode[a]	K_i (mM)[b]
p-Coumaric acid	Competitive	1.97
Kojic acid	Non-competitive	0.44
Ascorbic acid	Competitive	0.01
Sodium metabisulfite	Non-competitive	0.02
L-Cysteine	Uncompetitive	0.03[c]

[a] Determined from the Lineweaver–Burk plot of $1/V$ against 1/[guaiacol].

[b] Determined from the slope of the reciprocal plot against the inhibitor concentration.

[c] Determined from the *y*-intercept of the double reciprocal plot against the inhibitor concentration.

3.8. Effect of metal ions

The activities of mPOD isoenzymes from *M. sagu* were variously affected by the presence of metal ions. Al^{3+}, Ca^{2+}, Fe^{3+}, and Ni^{2+} stimulated the activity of mPOD-I better than mPOD-II (Table 5). On the other hand, 10 mM Mg^{2+}, Hg^{2+} and 1 mM Cu^{2+} did not show significant stimulation of either isoenzyme activity, whereas, 10 mM Zn^{2+} and Co+ moderately inhibited the activity of both isoenzymes. mPOD-I was more resistant to the inhibition effect by Zn^{2+} where the activity was reduced to 78%, compared to mPOD-II, which was reduced to 65%.

1 mM Fe^{3+} was able to greatly enhance the activity of both mPOD-I (339%) and mPOD-II (328%) (Table 5). Fe^{3+} is considered essential for the activity of most plant POD enzymes as it is involved in binding of H_2O_2 and formation of compound I (Whitaker, 1995; Wong, 1995). The presence of 10 mM Al^{3+} stimulates activity

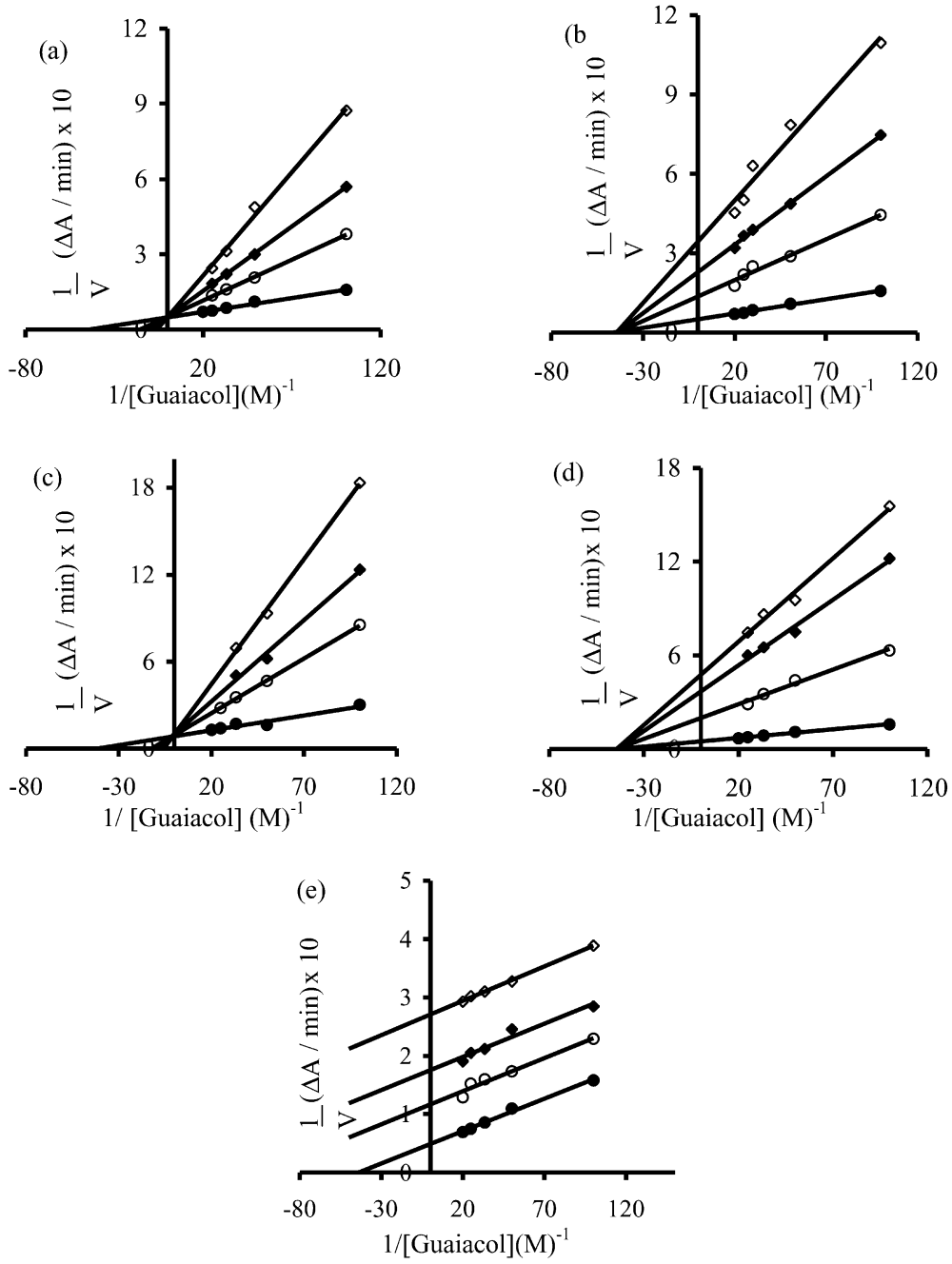

Fig. 7. Lineweaver double reciprocal plot of $1/V$ versus 1/[S] in the presence of three different fixed concentrations of different mPOD inhibitors: (a) *p*-coumaric acid (◇) 15.0 mM, (◆) 10.0 mM, (○) 5.0 mM and (●) without inhibitor. (b) Kojic acid (◇) 3.0 mM, (◆) 1.5 mM, (○) 0.5 mM and (●) without inhibitor. (c) Ascorbic acid (◇) 0.04 mM, (◆) 0.02 mM, (○) 0.01 mM and (●) without inhibitor. (d) Metabisulfite (◇) 0.04 mM, (◆) 0.02 mM, (○) 0.01 mM and (●) without inhibitor. (e) L-cysteine (◇) 0.15 mM, (◆) 0.1 mM (○) 0.05 mM and (●) without inhibitor.

of mPOD-I better (289%) than mPOD-II (216%). Among the metal ions tested, Ca^{2+} is a cofactor that serves to maintain the conformational integrity of the enzyme's active site (Adams et al., 1996; Robinson, 1991). Activation by Ca^{2+} ion was reported for avocado mPOD (Sanchez-Romera, Garcia-Gomez, Pliego-Alfero, & Heredia, 1994) and barley grain and wheat germ sPOD, where Ca^{2+} enhanced activities by ~2- and 6-fold, respectively (Billaud et al., 1999).

4. Conclusions

Temperature-induced phase partitioning was effectively used, for the first time, to isolate mPOD from *M. sagu,* in particular owing to the high starch density present. The study provides evidence for the occurrence of two isoenzymes of mPPO isolated from *M. sagu*. mPOD-I and mPOD-II from *M. sagu* were able to oxidize the phenolic compounds naturally present in sago

Table 5
Effects of selected metal ions on the activities of mPOD isoenzymes from *Metroxylon sagu*

Metal ion	Concentration (mM)	mPOD isoenzymes relative activity (%)	
		mPOD-I	mPOD-II
Control	0	100	100
$AlCl_3$	10	289	216
$NiCl_2$	10	117	113
CaCl	10	168	147
$ZnCl_2$	10	78	65
CoCl	10	95	92
$MgCl_2$	10	100	98
$HgCl_2$	1	100	100
$CuCl_2$	1	105	100
$FeCl_3$	1	339	328

log. This gives evidence of involvement of both mPOD isoenzymes in the enzymatic browning of *M. sagu*. Ascorbic acid, sodium metabisulfite, L-cysteine, kojic acid and *p*-coumaric acid, are food-compatible inhibitors that have proven high potential for inhibiting *M. sagu* mPOD.

Moreover, *M. sagu* mPPOs have pH optima of 6.0 and 5.5, for mPOD-I and mPOD-II, respectively, and they differ in prototropic groups at the active site. These variations in the kinetic properties of mPPO isoenzymes suggest that they carry multiple genes, each one encoding different types of enzyme units. Owing to the high molecular masses of 51.2 and 43.8 kDa, for mPOD-I and mPOD-II, respectively, it would be interesting to further investigate the carbohydrate structure of mPOD isoenzymes using *N*-glycan profiling and monosacchride composition analysis. This will provide better understanding of the structure-function relationships of mPODs.

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References

Adams, J. B., Harvey, A., & Dempsey, C. E. (1996). Regenerated and denatured peroxidase as potential lipid oxidation catalysis. *Food Chemistry*, *57*(4), 505–514.

Agostini, E., Medina, M. I., Milrad, S. R., & Tiger, H. (1997). Properties of two anionic peroxidase isoenzymes from turnip (*Brassica napus* L.) root. *Journal of Agricultural and Food Chemistry*, *45*, 596–598.

Ahmad, M. N. (1991). Optimizing starch granule extraction using a leaching prototype machine. In T. Thai-Tsiung, X. Yiu-Liong, & K. Honh-Siong (Eds.), *Proceedings of the 4th International Sago Symposium, August 1990* (pp. 188–193). Sarawak, Malaysia: Kuching.

Banci, L. (1997). Structural properties of peroxidases. *Journal of Biochemistry*, *53*, 253–263.

Belitz, H. D., & Grosch, W. (1999). Enzymes. In H. D. Belitz, & W. Grosch (Eds.), *Food chemistry* (21st ed) (pp. 92–139). Heidelberg, Washington, USA: Springer-Verlag.

Billaud, C., Louarme, L., & Nicolas, J. (1999). Comparison of peroxidases from barley kernel (*Hordeum vulgare* L.) and wheat germ (*Triticum aestivum* L.): isolation and preliminary characterization. *Journal of Food Biochemistry*, *23*, 145—172.

Boeuf, G., Bauw, G., & Rambour, S. (2000). Purification and characterization of a basic peroxidase from the medium of cell suspension culture of chicory. *Plant Physiology and Biochemistry*, *38*(3), 217–224.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-1 14 solution. *Journal of Biological Chemistry*, *256*(4), 1604–1607.

Boucoiran, C. F. S., Kijne, J. W., & Recourt, K. (2000). Isolation and partial characterization of thermostable peroxidases from potato (*Solanum tubarosum* L.) tuber sprouts. *Journal of Agricultural and Food Chemistry*, *48*, 701—707.

Chen, J. S., Wei, C., & Marshall, M. R. (1991). Inhibition mechanism of kojic acid on polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, *39*(11), 1897–1901.

Civello, P. M., Martinez, G. A., Chaves, A. R., & Anon, M. C. (1995). Peroxidase from strawberry fruit (*Fragaria ananassa* Duch.): partial purification and determination of some properties. *Journal of Agricultural and Food Chemistry*, *43*, 2596–25601.

Converso, D. A., & Fernandez, M. E. (1995). Peroxidase isozymes from wheat germ: purification and properties. *Phytochemistry*, *40*, 1341–1346.

Dixon, M., & Webb, D. C. (1979). Effect of pH. In D. Malcolm, Edwin C. Webb, C. J. R. Thorne, & K. F. Tipton (Eds.), *Enzymes* (3rd ed) (pp. 140–148). New York, USA: Longmans, Green Academic Press.

Duarte-Vazquez, M. A., Garcia-Almendarez, B., Regalado, C., & Whitaker, J. R. (2000). Purification and partial characterization of three turnip (*Brassica napus* L) peroxidases. *Journal of Agricultural and Food Chemistry*, *48*, 1574—1579.

Encyclopaedia Britannica. (1997). Sago and Sago starch. Available: http://www.britannica.comlbcomleb/article/0/0,5716,66416+64740,00.html.

Esteban-Carrasco, A., Zapata, J. M., Lopez-Serrano, M., Sabater, B., & Martin, M. (2002). Purification of two peroxidase isoenzymes of *Aloe barabadensis* which oxidize *p*-coumaric acid. *Plant Physiology and Biochemistry*, *40*, 127–132.

Flash, M. (1997). Sago palm *Metroxylon sagu*. In *Promoting the conservation and use of under-utilised and neglected crops*. (pp. 1–75). Rome, Italy: Institute of Plant Genetics and Crop Plant Research.

Forsyth, J. L., & Robinson, D. S. (1998). Purification of Brussels sprouts isoperoxidases. *Food Chemistry*, *63*(2), 227–234.

Friedman, M., & Bautista, F. F. (1995). Inhibition of polyphenol oxidase by thiols in the absence and presence of potato tissue suspensions. *Journal of Agricultural and Food Chemistry*, *43*, 69–76.

Fujita, S., Saari, N. B., Maegawa, M., Tetsuka, T., Hayashi, N., & Tono, T. (1995). Purification and properties of polyphenoloxidase from cabbage (*Brassica oleracea* L.). *Journal of Agricultural and Food Chemistry*, *43*, 1138–1142.

Gillikin, J. W., & Graham, J. S. (1991). Purification and developmental analysis of the major anionic peroxidase from seed coat of glycine max. *J. Plant Physiol.*, *96*, 214–220.

Gonzalez, E. M., Ancos, B., & Cano, M. P. (2000). Partial characterization of peroxidase and polyphenol oxidase activities in blackberry fruits. *Journal of Agricultural and Food Chemistry*, *48*, 5459–5464.

Hartree, E. F. (1972). Determination of protein: a modification of the

Lowry Method that gives a linear photometric response. *Analy. Biochem.*, *48*, 422–427.

Kato, M., Aibara, S., Morita, Y., Nakatani, H., & Hiromi, K. (1984). Transient Kinetics of peroxidase. *Journal of Biochemistry*, *95*(3), 861–869.

Lee, M. Y., & Kim, S. S. (1994). Characteristics of six isoperoxidases from Korean radish root. *Phytochemistry*, *35*(2), 287–290.

Leon, J. C., Alpeeva, I. S., Chubar, T. A., Galaev, I. Y., Csoregi, E., and Sakharov, I. Y. Purification and substrate specificity of peroxidase from sweet potato tubers. *Journal of Plant Science* (in press).

Leonardis, S., Dipierro, N., & Dipierro, S. (2000). Purification and characterization of an ascorbate peroxidase from potato tuber mitochondria. *Plant Physiology and Biochemistry*, *38*, 773–779.

Lin, Z., Chen, L., & Zhang, W. (1996). Peroxidase from *Ipomoea cairica* (L) SW. Isolation, purification and some properties. *Process Biochemistry*, *31*(5), 334–449.

Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society*, *56*, 658–666.

Lopez, A., & Burgos, J. (1995). Peroxidase stability and reactivation after heat treatment and manotherosonication. *Journal of Food Science*, *60*, 451–455.

Lopez-Serrano, M., & Barcelo, A. R. (1996). Purification and characterization of a basic peroxidase isozyme from strawberris. *Food Chemistry*, *55*(2), 133–137.

Melo, N. S., Cabral, J. M. S., & Fevereiro, M. P. (1995). Extracellular peroxidases from cell suspension cultures of *Vaccinium myrtillus*. Purification and characterization of two cationic enzymes. *Journal of Plant Science*, *106*, 177–184.

Moulding, P. H., Goodfellow, J., Mclellan, K. M., & Robinson, D. S. (1989). The occurrence of isoperoxidases in conference pear. *International Journal of Food Science and Technology*, *24*, 269–275.

Nicolas, J. J., Richard-Forget, F., Goupy, P., Amiot, M. J., & Aubert, S. (1994). Enzymatic Browning reactions in apple and apple products. *Critical Reviews of Food Science and Nutrition*, *34*(2), 109–157.

Onsa, G. H., Saari, N. B., Jinap, S., & Bakar, J. (2000). Latent polyphenoloxidases from sago log (*Metroxylon sagu*): partial purification activation and some properties. *Journal of Agricultural and Food Chemistry*, *48*, 5041–5046.

Padiglia, A., Pazzaglia, G., Cruciani, E., Medda, R., & Floris, G. (1995). Purification and characterization of opuntia peroxidase. *Phytochemistry*, *38*(2), 295–297.

Palmer, T. (1995). Kinetics of single-substrate enzyme catalysed reactions. In *Understanding Enzymes* (4th ed, pp. 107–127: Hertfordshire). Prentice Hall/Ellis Horwood.

Rasmussen, C. B., Henriksen, A., Abelskov, A. K., Jensen, B. J., Rasmussen, S. K., Hejgaard, J., & Welinder, K. G. (1997). Purification, characterization and stability of barley grain peroxidase BP 1, a new type of plant peroxidase. *Plant Physiology*, *100*, 102–110.

Regalado, C., Arvizu, P., & Garcia-Almendarez, B. (1999). Purification and properties of two acidic peroxidases from Brussels sprouts (*Brassica oleracea* L.). *Journal of Food Biochemistry*, *23*, 435–440.

Richard-Forget, F. C., & Gauillard, F. A. (1997). Oxidation of chlorogenic acid, catechins, and 4-methylcatechol in model solutions by combinations of pear (*Pyrus communis* Cv. Williams) polyphenol oxidase and peroxidase: a possible involvement of peroxidase in enzymatic browning. *Journal of Agricultural and Food Chemistry*, *45*, 2472–2476.

Riquelme, A., & Cardemil, L. (1993). Peroxidases in the cell walls of seeds and seedlings of Araucaria araucana. *Phytochemistry*, *32*(1), 15–20.

Robinson, D. S. (1991). Peroxidases and catalases in foods. In D. S. Robinson, & N. A. M. Eskin (Eds.), *Oxidative enzymes in food* (1st ed) (pp. 1–37). London & New York: Elsevier Applied Science.

Rodrique-Lopez, J. N., Espin, J. C., Amor, F., Tudela, J., Martinez, V., Cerda, A., & Garcia-Canovas, F. (2000). Purification and kinetic characterization of anionic peroxidase from melon *(Cucwnis mela* L.) cultivated under different salinity condition. *Journal of Agricultural and Food Chemistry*, *48*, 1537–1541.

Sakharov, I. Y., Vesga, M. K., Galaev, I. Y., Sakharova, I. V., & Pletushkina, O. Y. (2001). Peroxidase from leaves of royal palm tree *Roystonea regia*: purification and some properties. *Journal of Plant Science*, *161*, 853–860.

Sanchez-Romera, C., Garcia-Gomez, M., Pliego-Alfaro, F., & Heredia, A. (1994). Effect of partial deglycosylation on catalytic characterization and stability of an avocado peroxidase. *Physiologia Plantarum*, *92*, 97–101.

Scopes, R. K. (1993). Separation by adsorption II: Ion exchangers and nonspecific adsorbents. In R. K. Scopes, & C. R. Cantor (Eds.), *Protein purification: principles and practice* (3rd ed) (pp. 146–186). New York: Springer-Verlag.

Segel, I. H. (1976). Enzyme kinetic. In *Biochemical calculations* (2nd ed., pp. 214–245). John Wiley & Sons, Inc. Canada.

Segel, I. H. (1994). Simple inhibition system. In I. H. Segel (Ed.), *Enzyme kinetic; behavior and analysis of rapid equilibrium and steady-state enzyme systems* (pp. 100–136). Toronto, Canada: John Wiley and Sons.

Soda, I., Hasegawa, T., Suzuki, T., & Ogura, N. (1991). Purification and some properties of peroxidase from Kiwifruit. *Journal of Agricultural and Biological Chemistry*, *55*(6), 1677–1678.

Valero, E., Varon, R., & Garcia-Carmona, F. (1992). Kinetic study of the effect of metabisulfite on polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, *40*, 904–908.

Vamos-Vigyazo, L. (1981). Polyphenoloxidase and peroxidase in fruits and vegetables. *Critical Review of Food Science and Nutrition*, *15*, 49–127.

Voet, D., & Voet, J. G. (1990). Mechanisms of enzyme action. In Woolsey (Ed.), *Biochemistry* (1st ed) (pp. 344). New York: Viley.

Whitaker, J. R. (1994). Effect of pH on enzyme catalysed reactions. In *Principles of enzymology for the food science* (2nd ed.) (pp. 271–300). New York: Marcel Dekker.

Wong, D. W. (1995). Horseradish peroxidase. In W. S. Dominic (Ed.), *Food enzymes: structure and mechanism* (pp. 321–345). New York: Champman and Hall.

Zhou, P., Smith, N. L., & Lee, C. Y. (1993). Potential purification and some properties of Monroe apple peel polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, *41*(4), 1446–1449.