ELSEVIER

Food Chemistry 64 (1999) 351–359

Food Chemistry

A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. Romano)

Kiattisak Duangmal, Richard K. Owusu Apenten*

Procter Department of Food Science, School of Physical Sciences, University of Leeds, Leeds LS2 9JT, UK

Received 9 February 1998; received in revised form and accepted 1 May 1998

Abstract

Taro (*C. esculenta*) is a staple food in many tropical regions. A comparative study of crude polyphenoloxidases from taro (tPPO) and potatoes (pPPO) was carried out to provide information useful for guiding food processing operations. Crude PPO was prepared by cold acetone precipitation using ascorbic acid as antioxidant. The PPO content of taro acetone powder was 770 ± 17 units (mg protein)$^{-1}$ as compared with 3848 ± 180 units (mg protein)$^{-1}$ in potato acetone powder. The pH-activity optimum was pH 4.6 for tPPO and pH 6.8 for pPPO. Both enzymes retained $>80\%$ activity after incubation at pH 4.5–8 but there was rapid activity loss at pH < 4. The temperature-activity optimum (T_{opt}) was 30°C for tPPO and 25°C for pPPO with 75 and 27% of their respective maximum activity retained at 60°C. Both tPPO and pPPO were irreversibly inactivated by 10 min heating at 70°C. The activation enthalpy ($\Delta H^{\#}$) and activation entropy ($\Delta S^{\#}$) for tPPO heat-inactivation were 87.4 (± 0.1) kJ mol^{-1} and -56.2 (± 4) J mol^{-1} K^{-1}, respectively. For pPPO, $\Delta H^{\#}$ was 59.1 (± 0.1) kJ mol^{-1} whilst $\Delta S^{\#}$ was -141 (± 4) J mol^{-1} K^{-1}. The apparent substrate specificity was established from values V_{max}/K_m as: 4-methylcatechol > chlorogenic acid > DL-dopa > catechol > pyrogallol > dopamine > > caffeic acid for tPPO. There was no detectable activity towards caffeic acid. The substrate specificity for pPPO was: 4-methylcatechol > caffeic acid > pyrogallol > catechol > chlorogenic acid > DL-dopa > dopamine. According to the order of inhibitor effectiveness (sodium metabisulphite > ascorbic acid > NaCl $\approx$ (EDTA), there was a significant lag-phase before increases occurred in the absorbance at 420 nm. Preincubation of PPO with inhibitors increased the extent of inhibition, indicating a direct effect on the structure of the enzyme.

1. Introduction

Taro is a staple food in many tropical and subtropical areas of the world (Mega, 1992; Ravi et al., 1996). Taro corms may be processed, using techniques established for potato tubers (Mega, 1992). The polyphenoloxidase (PPO)-catalysed enzymatic browning reaction for taro needs to be characterised in order to facilitate its control during processing.

PPOs catalyse the oxidation of phenolic compounds to quinones. These further polymerise to brown pigments called melanins. PPO activity is widely distributed in fruits and vegetables. The action of PPO leads to enzymatic browning and major losses in some fresh fruits and vegetables, such as potatoes, lettuce, apples, apricots, bananas, grapes, peaches, strawberries and many other tropical fruits (cf. Vamos-Vigyazo, 1981; Zawistowski et al., 1991; Whitaker and Lee, 1995 for recent reviews). The PPO content depends on species, cultivars, maturity and age. Its distribution also varies with parts of fruits and vegetables (Anosike and Ayaebene, 1981; Lee et al., 1990; Asemato et al., 1992; Amiot et al., 1995). Several workers have studied PPO in potato (Patil and Zucker, 1965; Matheis, 1987a,b; Sapers et al., 1989), in sweet potato (Walter and Purcell, 1980; Lourenco et al., 1992), and in yam (Anosike and Ayaebene, 1981; Asemato et al., 1992).

PPO activities from other starch-rich tubers have not been well studied. Histological studies showing the distribution of taro polyphenoloxidase (tPPO) in intact tubers and their possible roles in chilling injury have been reported (Shiota, 1968; Rhee and Iwata, 1982). No substantive characterisation of tPPO has appeared in the recent literature.

In this work, characterisation of crude taro (*Colocasia esculenta*) PPO was studied and compared with the properties of crude potato (*Solanum tuberosum* var. Romano) PPO. Improved understanding of the characteristics of tPPO will lead to the prevention of browning during the processing of taro corm.

* Corresponding author. E-mail: r.k.owusu@leeds.ac.uk

0308-8146/98/$—see front matter
PII: S0308-8146(98)00127-7

2. Materials and methods

2.1. Plant materials and crude enzyme preparation

Taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. Romano) were purchased from a local market.

Peeled taro tuber (1 kg) was homogenised in 1 l of 0.1 M phosphate buffer (pH 7.0, 4°C) containing 30 mM ascorbic acid using a Waring blender. The homogenate was filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 13 000×*g* for 25 min (4°C) in a refrigerated centrifuge (Beckman model J2-HS, USA). The supernatant was then added to an equal amount of cold acetone (−30°C) and left for 20 min at 4°C to allow precipitation of the enzyme. The acetone precipitate was collected and dried over a Buchner funnel. The crude taro polyphenoloxidase (tPPO) was kept in a glass bottle in a freezer at −20°C. The same procedure was followed for the preparation of crude potato PPO (pPPO).

2.2. Protein determination

Determination of protein in crude taro and crude potato enzyme was done by the Lowry method as modified by Peterson (1977).

2.3. Enzyme assay

Taro acetone powder (70 mg) was dissolved in 7.0 ml of 0.1 M citrate–0.2 M phosphate buffer, pH 4.6. After being stirred for 5 min, the suspension was centrifuged at 12 000×*g* for 4 min. The supernatant was used as crude tPPO. For potato, 14 mg of acetone powder was dissolved in 7.0 ml of 0.1 M phosphate buffer, pH 6.8 instead.

PPO activity, assayed at 30°C, was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (Cecil instrument model CE 292, Cambridge, UK) fitted with a thermostated cuvette holder. The initial velocity was calculated from the slope of the absorbance-time curve. The reaction mixture contained 2.8 ml of 20 mM catechol in 0.1 M citrate–0.2 M phosphate buffer, pH 4.6 (20 mM catechol in 0.1 M phosphate buffer, pH 6.8, for pPPO activity) and 0.2 ml of enzyme solution. One unit of PPO activity was defined as the change in absorbance of 0.001 min^{-1}.

2.4. Properties of crude taro and potato PPOs

2.4.1. pH optima of crude PPO

The PPO activity from both sources was determined in a pH range of 3.2–6.4 in 0.1 M citrate–0.2 M phosphate buffer and in a pH range of 6.4–8.0 in 0.1 M phosphate buffer. PPO activity was assayed using catechol (20 mM) prepared in a buffer solution at various pH values as a substrate.

2.4.2. Temperature optima of crude PPO

tPPO activity was assayed at various reaction temperatures as controlled by a circulation water bath. The temperature was varied over the range of 6–74°C (6–59°C for pPPO). 4-Methylcatechol (5 mM) was used as a substrate.

2.4.3. pH stability of crude PPO

Enzyme solution (0.4 ml) was incubated for 30 min at 0°C in a buffer solution (0.8 ml) at various pH values. pH was varied over a pH range of 3–6 in 0.1 M citrate–0.2 M phosphate buffer and a pH range of 7–8 in 0.1 M phosphate buffer. 4-Methylcatechol (5 mM) was used as a substrate. PPO activity was determined in the form of percent residual PPO activity at the optimum pH.

2.4.4. Temperature stability of crude PPO

The enzyme solutions in Eppendorf tubes were incubated in a water bath at various temperatures (30, 40, 50, 60, 70°C) for 10 min. PPO activity was determined at 30°C, using 4-methylcatechol (5 mM) as a substrate. The percentage residual PPO activity was calculated by comparison with unheated enzyme.

2.4.5. Substrate specificity

tPPO activity was measured with seven substrates at various concentrations according to their solubility: 2.5, 5, 10, 20, and 40 mM for 4-methylcatechol, catechol, pyrogallol, and dopamine; 2.5, 5, 10, 15, and 20 mM for DL-dopa; 1.25, 2.5, 5, 8, and 10 mM for chlorogenic acid and caffeic acid. Substrates were dissolved in 0.1 M citrate–0.2 M phosphate buffer, pH 4.6. Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enzyme were calculated from a plot of 1/v vs 1/[S] by the method of Lineweaver and Burk. For pPPO activity, each substrate was dissolved in 0.1 M phosphate buffer, pH 6.8.

2.4.6. Effect of inhibitors on PPO

Inhibitors examined included EDTA, NaCl, ascorbic acid, and sodium metabisulphite. For crude tPPO activity, the reaction mixture contained 2.6 ml of 5 mM 4-methylcatechol in 0.1 M citrate–0.2 M phosphate buffer (pH 4.6), 0.2 ml of enzyme and 0.2 ml of inhibitor solution. Concentration of inhibitors varied with their inhibitory effects: 0, 10, 20, 30, and 40 mM for EDTA and NaCl; 0, 1, 2.5, 5, and 10 mM for ascorbic acid; 0, 0.5, 1, and 2.5 mM for sodium metabisulphite. For crude pPPO activity, the reaction mixture contained 2.6 ml of 5 mM 4-methylcatechol in 0.1 M phosphate buffer (pH 6.8), 0.2 ml of enzyme and 0.2 ml of inhibitor solution. The concentration of inhibitors varied with their inhibitory effects: 0, 10, 20, 30, and 40 mM for

EDTA and NaCl; 0, 1, 2.5, and 5 mM for ascorbic acid; 0, 0.5, 1, 2.5, and 5 mM for sodium metabisulphite. The percentage relative inhibition for each inhibitor was calculated as in Eq. (1). Two different conditions: pre-incubation of enzyme in inhibitor solution for 10 min and without pre-incubation of enzyme in inhibitor solution were studied. The percentage inhibition is expressed as

$$[(A_O - A_I)/A_O] \times 100 \quad (1)$$

where A_O is activity of uninhibited enzyme and A_I is activity of inhibited enzyme.

3. Results and discussion

The current results were derived from a study of crude enzyme preparations extracted from acetone powder. The properties of the crude, as opposed to a purified, enzyme preparation may well be derived from a combination of isoenzymes. There may also be interactions with nonenzymatic proteins. Due to their complex nature, it is usual to refer to the characteristics of crude enzyme preparations as 'apparent' quantities. The properties described below are therefore apparent quantities even where the epitaph (apparent) has not been used. However, the properties of a crude enzyme preparation can be as relevant to the Food industry as those of the purified or isolated enzyme.

3.1. pH optima of crude PPO activity

The pH activity profiles for crude taro polyphenol oxidase (tPPO) and crude potato polyphenol oxidase (pPPO) are shown in Fig. 1. tPPO had a maximum activity around pH 4.6 and a secondary maximum activity around pH 7.0. On the other hand, pPPO had a maximum activity around pH 6.8 with a narrow plateau between pH 4.4 and 6.0. As can be seen from the graph, the PPO from both sources were nearly inactive at pH 4.0. Differences in the pH optimum for pPPO have been reported by several workers (Alberghina, 1964; Patil and Zucker, 1965; Abukharma and Woolhouse, 1966; Matheis, 1987a). The optimum pH depends on genetic properties (variety), nature of phenolic substrates and extraction methods. The pH optimum for tPPO does not appear to have been reported before.

3.2. Temperature optima of crude PPO activity

The temperature activity profiles for crude tPPO and pPPO are shown in Fig. 2. The optimum temperature for activity of tPPO was at 30°C while that of pPPO was at 25°C. At an assay temperature 30°C above the optimum temperature of each enzyme, 25% of tPPO activity was lost whereas 65% of pPPO activity was lost. It appears that crude pPPO is more sensitive to the increase in assay temperature than crude tPPO. The optimum temperature of pPPO was reported to be 22°C when catechol was used as a substrate whereas it was reported to be 35°C when pyrogallol was used as a substrate (Matheis, 1987a).

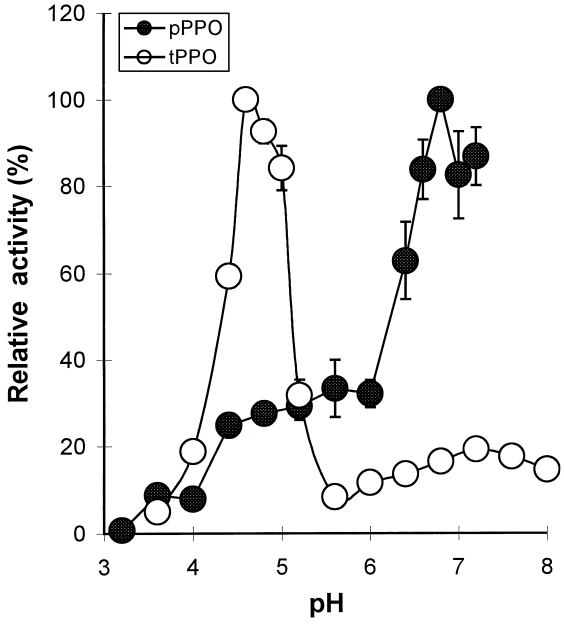

Fig. 1. pH activity profiles for taro PPO (○) or potato PPO (●) in 0.1 M citrate–0.2 M phosphate buffer (pH 3.2–6.4) and in 0.1 M phosphate buffer (pH 6.4–8).

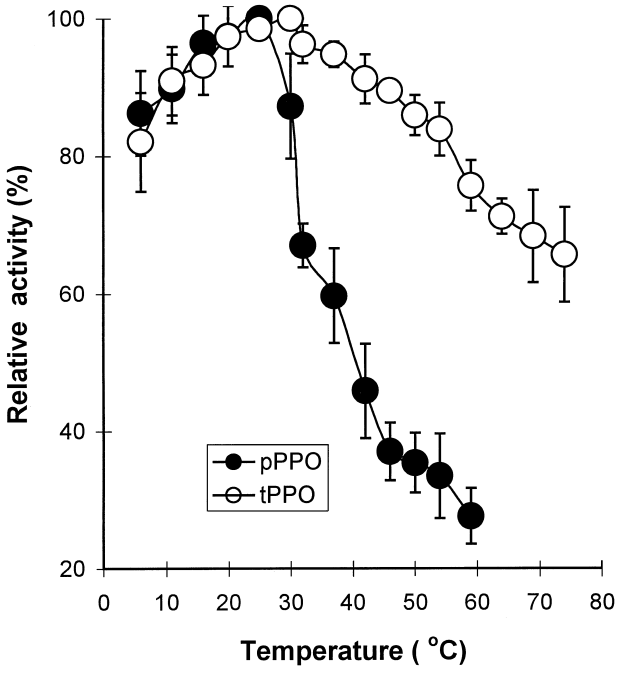

Fig. 2. Temperature activity profiles for taro PPO (○) in 0.1 M citrate–0.2 M phosphate buffer (pH 4.6) and potato PPO (●) in 0.1 M phosphate buffer (pH 6.8).

3.3. pH stability of crude PPO

The residual percentage activity of the enzyme from both sources for various pH values between 3 and 8 is shown in Fig. 3. The pH-stability profiles for both enzymes were quite similar. There was >80% retention of activity at pH 4.5–8.0. Both enzymes were not stable at pH below 4.

3.4. Temperature stability of crude PPO

Temperature-stability profiles for crude tPPO and pPPO, presented in the form of the residual percentage activity, are shown in Fig. 4(a). Although both enzymes shared similar temperature stability profiles, the drop in percentage residual activity of crude pPPO at high temperature was greater than that of crude tPPO. A considerable drop in PPO activity at high temperature is probably the result of changes in the enzyme tertiary structure. Heating at 70°C for 10 min completely inactivated both tPPO and pPPO. Heating at 60°C for the same duration resulted in partial (60–70%) inactivation. PPO is not considered a heat-stable enzyme. Short exposures to temperatures of 70–90°C are sufficient for partial or total irreversible destruction of its catalytic activity (Vamos-Vigyazo, 1981).

The activation energy ($\Delta E^{\#}$), enthalpy ($\Delta H^{\#}$), entropy ($\Delta S^{\#}$) and free energy change ($\Delta G^{\#}$) for crude tPPO or pPPO heat-inactivation were determined from results in Fig. 4(a). Assuming that PPO inactivation conforms to apparent first order kinetics, the rate constant for heat-inactivation (k) was determined from the relation below

$$k = -(1/600).\ \ln (A_t/A_o) \quad (2)$$

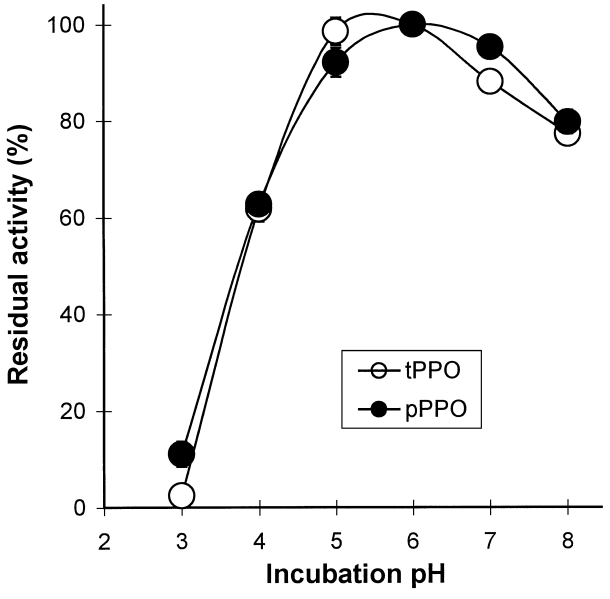

Fig. 3. pH-stability profiles for taro PPO (○) and potato PPO (●).

where A_t is the residual enzyme activity remaining after heating for 600 s at various temperatures and A_o is the activity of unheated enzyme. The temperature-dependence of k (s^{-1}) was evaluated using the Arrhenius equation,

$$\ln (k) = C - {}^{\#}/RT \quad (3)$$

as shown by the graph in Fig. 4(b).

Other activation parameters were determined from the relations below as described previously (Amiza and Apenten, 1994; Galani and Apenten, 1997):

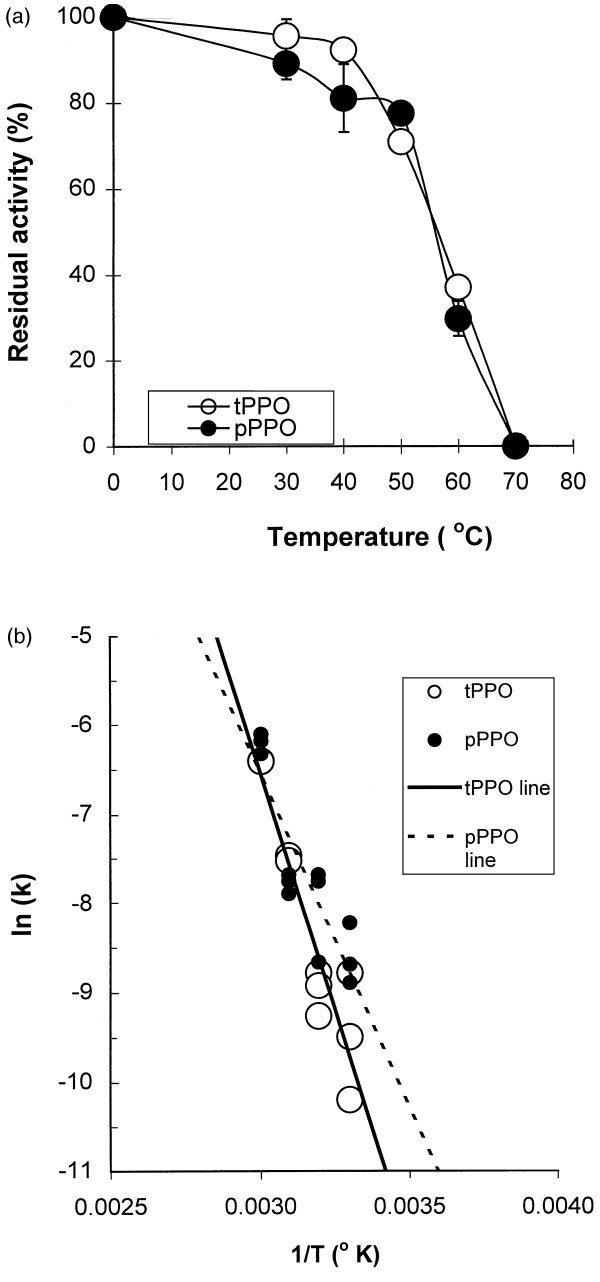

Fig. 4. (a) Heat inactivation profile for taro PPO (○) and potato PPO (●). (b) Arrhenius plot for heat inactivating of taro PPO (○) and potato PPO (●).

$$= RT\ln(kT/Kh) \quad (3a)$$

$$^{\#} = ^{\#} - RT \quad (3b)$$

$$^{\#} = (^{\#} - ^{\#})/T \quad (3c)$$

where R (8.3145 J mol^{-1} °K^{-1}) is the universal gas constant, K (1.3806×10^{-23} J °K^{-1}) is the Boltzman constant, and h (6.6261×10^{-34} J s) is the Planck constant. Results for these analyses are reported in Table 1.

To summarise, $\Delta E^{\#}$ was 90.1 kJ mol^{-1} for tPPO heat inactivation as compared to 61.8 kJ mol^{-1} for pPPO heat inactivation. At temperatures of 30–70°C, the average values of $\Delta H^{\#}$ were 87.4 (±0.1) kJ mol^{-1} and 59.1 (±0.1) kJ mol^{-1} for tPPO and pPPO heat inactivation, respectively. The value of $\Delta S^{\#}$ was −56.2 (±4.1) J mol^{-1} °K^{-1} for tPPO inactivation and −142 (±3.9) J mol^{-1} °K^{-1} for pPPO inactivation.

The preceding activation parameters are consistent with the view that PPOs are relatively heat-labile enzymes. Results from Table 1 suggest that tPPO is slightly more heat-resistant than pPPO, apparently as a result of the larger $\Delta H^{\#}$ value for inactivation. Based on $\Delta S^{\#}$ values alone, pPPO would have been more heat-resistant than tPPO. In general, $\Delta H^{\#}$ is seen as a measure of the number of noncovalent bonds broken in forming a transition state for enzyme inactivation. $\Delta S^{\#}$ is measure of the net enzyme and solvent disorder change accompanying transition state formation.

3.5. Substrate specificity

Apparent K_m and V_{max} values for crude taro and crude pPPO are presented in Table 2. The value for K_m is normally constant for each particular substrate and enzyme while V_{max} varies with the concentration of enzyme. The best substrate for each enzyme depends on two factors: strong substrate binding as expressed by a low K_m and high catalytic efficiency, as expressed by a high V_{max} value (for a fixed enzyme concentration). Thus, the criterion for the best substrate is the V_{max}/K_m ratio (Palmer, 1995).

From the apparent V_{max}/K_m values (Table 2), the best substrate is 4-methylcatechol for both tPPO and pPPO. Abukharma and Woolhouse (1966) have reported that 4-methyl catechol was the preferred substrate for pPPO from the King Edward variety of potato. Walker (1995) has also noted that 4-methylcatechol is usually the best substrate for plant PPOs.

tPPO and pPPO were both able to catalyse the oxidation of dopamine. However, for this substrate K_m and V_{max} cannot be calculated from a plot of 1/v vs 1/[S] by the method of Lineweaver and Burk.

The amount of soluble protein in taro and potato acetone powders was about 17 and 4%, respectively. The amount of PPO in taro and potato acetone powders, was estimated from V_{max} values

Table 1
Transition state parameters for the heat inactivation of crude taro PPO and potato PPO

Temp. Parameter	$\Delta E^{\#}$ (J mol^{-1})	$\Delta H^{\#}$ (J mol^{-1})	$\Delta G^{\#}$ (J mol^{-1})	$\Delta S^{\#}$ (J mol^{-1} °K^{-1})
Taro PPO				
30°C	90095.26	87575.96	104280.15	−55.13
40°C	90095.26	87492.82	106226.69	−59.85
50°C	90095.26	87409.67	105542.70	−56.14
60°C	90095.26	87326.53	105794.20	−55.46
Mean [a]	90095.3	87451.2	—[b]	−56.2
SD	9270	97.1		4.1
Potato PPO				
30°C	61830.78	59311.49	102656.79	−143.05
40°C	61830.78	59228.34	105489.54	−147.80
50°C	61830.78	59145.20	106746.56	−147.37
60°C	61830.78	59062.05	105556.59	−139.62
Mean [a]	61830.8	59186.8	—[b]	−141.9
SD	10077.0	97.1		3.9

[a] Mean (±SD) for 12 estimates from triplicate experiments.
[b] Parameter varies with temperature (cf. Eq. (3)(a)).

Table 2
Apparent K_m[a] and V_{max}[b] values with different substrates for crude taro PPO and potato PPO

Substrate and parameter	Crude taro PPO	Crude pPPO
4-Methylcatechol		
K_m (mM)	9.0±0.4	1.1±0.1
V_{max} (units/mg protein)	770.0±17.3	3848.7±180.5
V_{max}/K_m	85.6	3498.8
Catechol		
K_m (mM)	67.9±7.1	6.8±0.4
V_{max} (units/mg protein)	849.0±87.4	9453.3±344.3
V_{max}/K_m	12.5	1390.2
Pyrogallol		
K_m (mM)	80.9±8.6	1.5±0.1
V_{max} (units/mg protein)	352.3±31.9	2247.3±119.8
V_{max}/K_m	4.4	1498.2
Chlorogenic acid		
K_m (mM)	2.3±0.2	1.7±0.1
V_{max} (units/mg protein)	163.0±6.2	1655.3±97.0
V_{max}/K_m	70.9	973.7
Caffeic acid		
K_m (mM)	0	2.3±0.3
V_{max} (units/mg protein)	0	3893.3±312.3
V_{max}/K_m	—	1692.7
DL-dopa		
K_m (mM)	17.0±2.0	9.4±0.9
V_{max} (units/mg protein)	273.0±25.0	2653.7±296.8
V_{max}/K_m	16.1	282.3
Dopamine		
K_m (mM)	[b]	[b]
V_{max} (units/mg protein)	[b]	[b]
V_{max}/K_m	—	—

[a] Values are reported as mean±S.D. for triplicate experiments.
[b] Inappropriate substrate, K_m and V_{max} cannot be calculated from a plot of 1/v vs 1/[S] by the method of Lineweaver and Burk.
One unit of PPO activity produces an A_{420} change of 0.001 min^{-1}.

using 4-methylcatechol as a substrate, as 131×10^3 units g^{-1} (taro acetone powder) and 154×10^3 units g^{-1} (potato acetone powder).

3.5.1. Effect of inhibitors on crude PPO

The effects of four inhibitors, namely EDTA, NaCl, ascorbic acid, and sodium metabisulphite on crude tPPO and pPPO activity were studied. The results are shown in Figs. 5 and 6. The percentage inhibition was compared with that of the control (100% activity). Sodium metabisulphite was the most effective inhibitor followed by ascorbic acid. The effect of each inhibitor on tPPO and pPPO is discussed below. The general mechanisms of PPO inhibition have been reviewed by Iyengar and McEvily (1992), Sapers (1993) and Martinez and Whitaker (1995).

3.5.2. Effect of EDTA on crude PPO

As seen from Figs. 5(A) and 6(A), the effect of EDTA on crude tPPO activity was similar to that on crude pPPO activity. The PPO activity decreased as the concentration of EDTA increased. However, EDTA was not a good inhibitor. At any level of EDTA concentration, enzyme activities obtained from pre-incubation conditions were lower than those obtained from without pre-incubated condition. These could be explained by the difference in contact time. With increased contact time, EDTA could react to a greater extent with the enzyme to suppress PPO activity. We expect a definite effect from pre-incubation if the inhibitor acts directly on the enzyme structure.

It is believed that EDTA can form a complex with Cu^{2+} in PPO. This formation leads to a decrease in enzyme activity. The inhibitory effect of EDTA would depend on the binding constant for the complex formed between metal ion and EDTA compared to the binding constant for the complex of metal ion with the enzyme (Whitaker, 1994). It has been reported by several workers that EDTA is not a good PPO inhibitor. Luh and Phithakpol (1972) found that the effect of EDTA on cling peaches PPO activity was quite low. They had speculated that the pH of the reaction mixture (pH 6.2) may affect the affinity of EDTA towards copper which is an important part of PPO; therefore, EDTA showed a

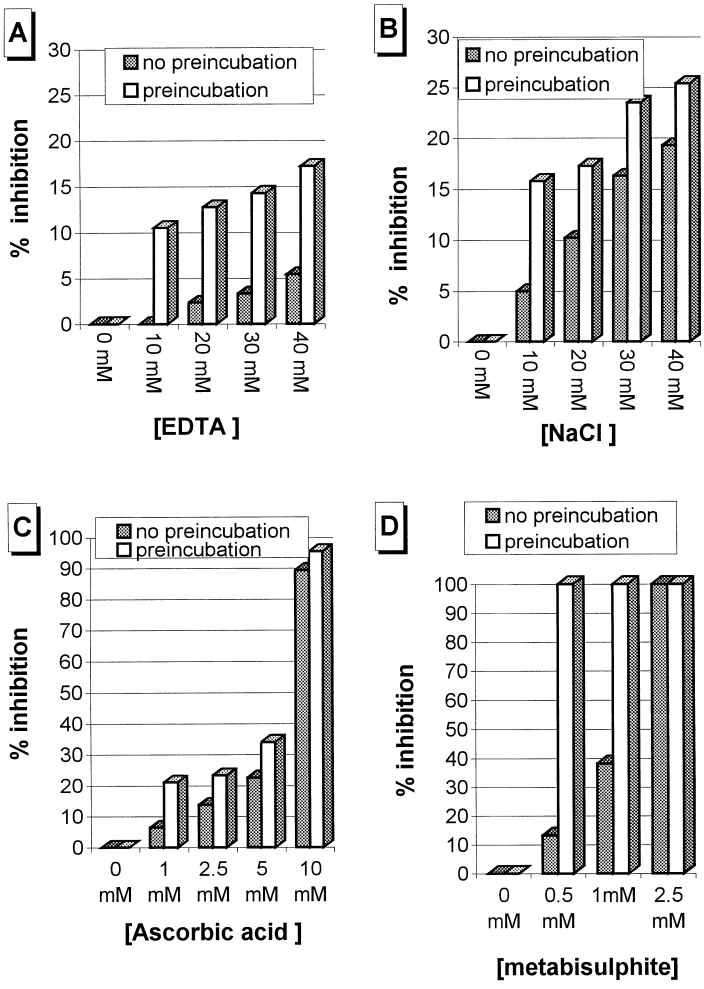

Fig. 5. Effect of inhibitors on taro PPO in 0.1 M citrate–0.2 M phosphate buffer (pH 4.6). Inhibitors were; (A) EDTA, (B) NaCl, (C) ascorbic acid and (D) sodium metabisulphite.

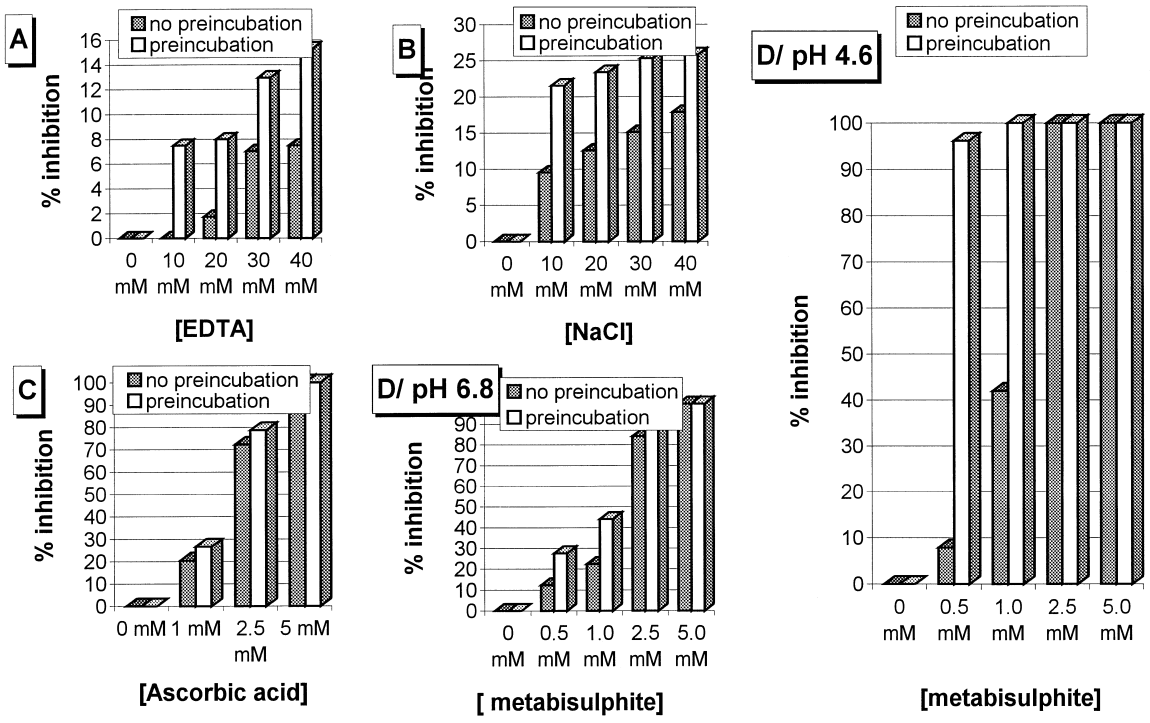

Fig. 6. Effect of inhibitors on potato PPO in 0.1 M phosphate buffer (pH 6.8). Inhibitors were; (A) EDTA, (B) NaCl, (C) ascorbic acid (D) sodium metabisulphite and (E) sodium metabisulphite in 0.1 M citrate–0.2 M phosphate buffer (pH 4.6).

slight effect on PPO inhibition. The effect of EDTA on purified yam PPO activity, studied by Anosike and Ayaebene (1981), was slight. In addition, Lamikanra (1995) claimed that EDTA had no significant effect on the rate of colour development in Muscadine wine.

3.5.3. *Effect of NaCl on crude PPO*

The activity of PPO decreased as the concentration of NaCl increased (Figs. 5(B) and 6(B)). The effect of NaCl on crude tPPO activity was similar in pattern to that of crude pPPO activity. Compared to the effect of EDTA, the effect of NaCl on the inhibition of enzyme activity was slightly greater. However, the highest concentration of NaCl used, 40 mM, showed less than 30% relative inhibition compared to the control. From the data, it may be concluded that the inhibitory effect of NaCl was not satisfactory. This may be the result of the low concentration of NaCl. At any level of NaCl concentration, PPO activities obtained from pre-incubation conditions were lower than those obtained from no pre-incubation condition. Nonetheless, the differences between both conditions were only slight.

It is believed that the action of NaCl is due to its interaction with the copper at the active centre of the enzyme (Iyengar and McEvily, 1992; Sapers, 1993; Martinez and Whitaker, 1995). Several works have been reported on the effect of NaCl on PPO activity. Luh and Phithakpol (1972) claimed that a 3% sodium chloride solution (0.51 M) may be used as an inhibitor to hinder enzymatic browning in cling peaches. On the other hand, the inhibitory effect at low NaCl concentration on PPO activity is quite low. It was shown that the inhibition of PPO from d'Anjou pears by 10 mM NaCl was 12%; by 1 mM NaCl it was 8% compared to the control which contained no NaCl (Halim and Montgomery, 1978). Similar findings were also reported by Benjamin and Montgomery (1973) on cherry PPO. In comparison with the control, the inhibition of PPO from cherry by 400 mM NaCl was 18%.

3.5.4. *Effect of ascorbic acid on crude PPO*

As shown in Fig. 5(C), at low ascorbic acid concentration, the inhibitory effects on crude tPPO were slight (with 1, 2.5 and 5 mM ascorbic acid there was 21, 23 and 34% inhibition, respectively), while the result obtained at higher ascorbic acid concentration (10 mM) was satisfactory (nearly 100%). The effect of ascorbic acid on crude pPPO activity is shown in Fig. 6(C). The inhibitory effect increased as the concentration of ascorbic acid increased. It can be seen that pPPO tends to be inhibited by ascorbic acid more easily than tPPO (cf. Figs. 5(C) and 6(C)).

At a given ascorbic acid concentration, there were no significant differences between the degree of PPO inhibition obtained with or without pre-incubation. This is as expected provided that ascorbic acid does not act directly on the enzyme structure (see below). From the above information, it can be concluded that ascorbic

acid is an effective inhibitor of crude tPPO and pPPO. This view is supported by results from Halim and Montgomery (1978) who found that the inhibition of d'Anjou pears PPO was 100% when 10 mM ascorbic acid was used.

In PPO assays, where ascorbic acid was used as an inhibitor, a lag period was observed before any changes in absorbance were measured. The extent of the lag period increased with an increase in inhibitor concentration. Similar findings were also reported by Halim and Montgomery (1978), Lourenco et al. (1992) and Ngalani et al. (1993). The mechanism of ascorbic acid inhibition involves the reduction of quinones generated by PPO (Iyengar and McEvily, 1992; Sapers, 1993; Whitaker, 1994; Martinez and Whitaker, 1995; Whitaker and Lee, 1995). PPO catalyses the oxidation of phenolic substrates to *o*-quinones whilst ascorbic acid converts *o*-quinones back to phenolic compounds. Changes in absorbance ($A_{420\,nm}$) cannot be observed at the beginning of the reaction, thus creating a lag period. After the lag period, when nearly all ascorbic acid is converted to dehydroascorbic acid, the amount of *o*-quinones formed by action of PPO increases. The *o*-quinones then polymerise and or combine together with amino compounds to form high molecular weight brown pigments.

3.5.5. *Effect of sodium metabisulphite on crude PPO*

From the data in Figs. 5(D) and 6(D), sodium metabisulphite was the most effective PPO inhibitor. The action of sulphite in the prevention of enzymatic browning can usually be explained by several processes. One is the action on *o*-quinones. The formation of quinone-sulphite complexes prevents the quinone polymerisation (Embs and Markakis, 1965). A further action of metabisulphite on PPO is directly on the enzyme structure leading to the inactivation of PPO (Golan-Goldhirsch and Whitaker, 1984). The latter effect is indicated by the difference between tPPO inhibition obtained with and without pre-incubation. Embs and Markakis (1965) and Golan-Goldhirsch and Whitaker (1984) found that, during pre-incubation of PPO with sulphite (dithreitol, gluthathione or ascorbic acid), there was a gradual loss in the ability of the enzyme to cause browning.

It has been suggested that sulphite reacts with disulphide bonds within PPO. This leads to the change in tertiary structure of enzyme and inactivation. Compared to the effect of sodium metabisulphite on tPPO, that effect on pPPO was not strong. This difference was identified as arising from the effect of pH on the action of sodium metabisulphite.

tPPO inhibition was initially tested at pH 4.6 whereas pPPO inhibition was studied at pH 6.8. These pH values corresponded to the pH optimum for each enzyme. In order to confirm the postulate that the pH of inhibition is important, pPPO solutions were prepared in acidic pH (pH 4.6) and pre-incubated with metabisulphite at pH 4.6. The residual PPO activity was then determined at pH 6.8. The result showed greatly enhanced inhibition of pPPO by metabisulphite at low pH.

In aqueous solution, soluble sulphites, as well as SO_2, exist mainly as a mixture of the ionic species sulphite (SO_3^{2-}) and bisulphite (HSO_3^-). The relative proportion of each form depends on the pH of the solution. At pH 4.6 the bisulphite anion is predominant and at pH 7 both species exist in the same proportion. Sayavedra-Soto and Montgomery (1986) showed, with pre-incubation of PPO in sulphite, that the PPO inhibition using sulphite was irreversible. Increasing sulphite concentrations or lowering the solvent pH (to below pH 5) enhanced PPO inhibition. In addition, they also proved that HSO_3^- was the main component in the sulphite system inhibiting PPO. The major mode for the irreversible inhibition was via the modification of PPO structure. When tPPO or pPPO was pre-incubated in $\geq$2.5 mM sodium metabisulphite for 10 min, there was complete inactivation.

During intial velocity measurements for tPPO or pPPO in the presence of sodium metabisulphite, a lag period was observed before changes in A_{420} readings occurred (data not shown). The extent of the lag period increased with an increase in sodium metabisulphite concentration. The creation of a lag period during PPO assays is usually a feature of the PPO inhibitors which act as reducing agents. The third process leading to PPO inhibition by bisulfite is via reduction of the intermediate quinones as described for ascorbic acid.

Acknowledgements

The authors are grateful to the Royal Thai government for providing a research scholarship to KD.

References

Abukharma, D. A. and Woolhouse, H. U. (1966) The preparation and properties of *o*-diphenol: oxygen oxidoreductase from potato tubers. *New Phytol.* **65**, 477–478.

Alberghina, F. A. M. (1964) Chlorogenic acid oxidase from potato tuber slices: partial purification and properties. *Phytochem.* **3**, 65–72.

Amiot, M. J., Tacchini, M., Aubert, S. Y. and Oleszek, W. (1995) Influence of cultivars, maturity stage, and storage conditions on phenolic composition and enzymatic browning of pear fruits. *J. Agric. Food Chem.* **43**, 1132–1137.

Amiza, M. A. and Apenten, R. K. O. (1994) Thermal inactivation parameters for alkaline proteinases from North Sea cod (*Gadus morhua*) and α-chymotrypsin. bovine. *J. Sci. Food. Agric.* **66**, 389–391.

Anosike, E. O. and Ayaebene, A. O. (1981) Purification and some properties of polyphenoloxidase from the yam tubers, Dioscorea bulbifera. *Phytochem.* **20**(12), 2625–2628.

Asemato, H.N., Wellington, M.A., Odutuga, A.A. and Ahmad, M.H. (1992) Effect of short-term storage on phenolic content, o-diphenolase and peroxidase activities of cut yam tubers (*Dioscorea* sp). *J. Sci. Food Agric.* **60**, 309–312.

Benjamin, N.D. and Montgomery, M.W. (1973) Polyphenol oxidase of Royal Ann cherries: purification and characterisation. *J. Food Sci.* **38**, 799–806.

Embs, R.J. and Markakis, P. (1965) The mechanism of sulphite inhibition of browning caused by polyphenol oxidase. *J. Food Sci.* **30**, 753–758.

Galani, D. and Apenten, R.K.O. (1997) The comparative heat stability of bovine β-lactoglobulin in buffer and complex media. *J. Sci. Food Agric.* **74**, 89–98.

Golan-Goldhirsch, A. and Whitaker, J.R. (1984) Effect of ascorbic acid, sodium bisulfite, and thiol compounds on mushroom polyphenol oxidase. *J. Agric. Food Chem.* **32**(5), 1003–1009.

Halim, D. H. and Montgomery, M. W. (1978) Polyphenoloxidase of d'Anjou pears (*Pyrus communis* L.). *J. Food Sci.* **43**, 603–606, 608.

Iyengar, R. and McEvily, A. J. (1992) Anti-browning agents: alternatives to the use of sulfites in foods. *Trends Food Sci. Technol.* **3**(3), 60–64.

Lamikanra, O. (1995) Enzymatic browning of Muscadine grape products. In *Enzymatic Browning and Its Prevention*, ed. Y. L. Chang and J. R. Whitaker, pp. 166–177. ACS Symposium Series 600. American Chemical Society, Washington, DC.

Lee, C. Y., Kagan, V., Jaworski, A. W. and Brown, S. K. (1990) Enzymatic browning in relation to phenolic compounds and polyphenoloxidase activity among various peach cultivars. *J. Agric. Food Chem.* **38**, 99–101.

Lourenco, E. J., Neves, V. A. and Da Silva, M. A. (1992) Polyphenol oxidase from sweet potato: purification and properties. *J. Agric. Food Chem.* **40**, 2369–2373.

Luh, B. S. and Phithakpol, B. (1972) Characteristics of polyphenoloxidase related to browning in cling peaches. *J. Food Sci.* **37**, 264–268.

Martinez, M. and Whitaker, J. R. (1995) The biochemistry and control of enzymatic browning. *Trends Food Sci. Technol.* **6**(6), 195–200.

Matheis, G. (1987a) Polyphenol oxidase and enzymatic browning of potatoes (*Solanum tuberosum*) I. Properties of potato polyphenol oxidase. *Chem. Mikrobiol. Technol. Lebensm.* **11**, 5–12.

Matheis, G. (1987b) Polyphenol oxidase and enzymatic browning of potatoes (*Solanum tuberosum*) II. Enzymatic browning and potato constituents. *Chem. Mikrobiol. Technol. Lebensm.* **11**, 33–41.

Mega, J. A. (1992) Taro: composition and food uses. *Food Review Int.* **8**, 443–473.

Ngalani, J. A., Signoret, A. and Crouzet, J. (1993) Partial purification and properties of plantain polyphenol oxidase. *Food Chem.* **48**, 341–347.

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

Patil, S. S. and Zucker, M. (1965) Potato phenolases: purification and properties. *J. Biol. Chem.* **240**(10), 3938–3943.

Peterson, G. L. (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**, 346–356.

Ravi, V., Aked, J. and Balagopalan, C. (1996) Review on tropical root and tuber crops I. Storage methods and quality changes. *Crit. Rev. in Food Nutr.* **36**(7), 661–709.

Rhee, J. K. and Iwata, M. (1982) Histological observations on the chilling injury of taro tubers during cold storage. *J. Jpn. Soc. Hort. Sci.* **51**(3), 362–368.

Sapers, G. M. (1993) Browning of foods: control by sulfites, antioxidants, and other means. *Food Technol.* **47**(10), 75–81.

Sapers, G. M., Douglas, Jr, F. W., Bilyk, A., Hsu, A. F., Dower, H. W., Garzarella, L. and Kozempel, M. (1989) Enzymatic browning in Atlantic potatoes and related cultivars. *J. Food Sci.* **54**(2), 362–365.

Sayavedra-Soto, L. A. and Montgomery, M. W. (1986) Inhibition of polyphenoloxidase by sulphite. *J. Food Sci.* **51**, 1531–1536.

Shiota, Y. (1968) Browning of peeled tubers of taro (*Colocasia esculenta*). *J. Food Sci. Technol. (Japan)* **15**(12), 547–552.

Vamos-Vigyazo, L. (1981) Polyphenol oxidase and peroxidase in fruits and vegetables. *CRC Crit. Rev. Food. Nutr.* **15**, 49–127.

Walker, J. R. L. (1995) Enzymatic browning in fruits: its biochemistry and control. In *Enzymatic Browning and Its Prevention*, ed. Y. L. Chang and J. R. Whitaker, pp. 8–22. ACS Symposium Series 600. American Chemical Society, Washington, DC.

Walter, Jr W. M. and Purcell, E. A. (1980) Effect of substrate levels and polyphenol oxidase activity on darkening in sweet potato cultivars. *J. Agric. Food Chem.* **28**, 941–944.

Whitaker, J. R. (1994) Enzyme inhibitors. *Principles of Enzymology for the Food Sciences.* 2nd edn, pp. 241–270. Marcel Dekker, Inc. New York.

Whitaker, J. R. and Lee, C. Y. (1995) Recent advances in chemistry of enzymatic browning: An overview. In *Enzymatic Browning and Its Prevention*, ed. Y. L. Chang and J. R. Whitaker, pp. 2–7. ACS Symposium Series 600. American Chemical Society, Washington, DC.

Zawistowski, J., Biliaderis, C. G. and Eskin, N. A. M. (1991) Polyphenol oxidase. In *Oxidative Enzymes in Foods*, ed. D. S. Robinson and M. A. N. Eskin, pp. 217–273. Elsevier Applied Science Ltd, London.