# Characterization and Comparative Study of Polyphenol Oxidases from Four Cultivars of Thai *Solanum melogena* Fruits

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## ABSTRACT

***This study characterized the polyphenol oxidases (PPO) from four cultivars of eggplant (Thai Solanum melogena), representing a variety of fruit characteristics, textures, and peel colors. The study compared the effects of different substrate specificity, temperature, pH, thermal stability, and inhibitors on PPO activity to determine the optimal conditions for generating natural browning agents. Khaow yoa (OW) and Moung khan khew (OP) cultivars had the highest specific activity (OW: 631.33 ± 38.29 ΔOD $min^{-1}$ mgProtein-1; OP: 652.54 ± 9.59 ΔOD $min^{-1}mgProtein^{-1}$), using 4-methylcatechol as a substrate. The best substrate for PPO from each cultivar was different: catechin for cultivars OW and OP, 4-MC for Choa pra ya (RG), and 4-tert butylcatechol for Khaew yoa (OG). Using 4-methylcatechol as a substrate, the optimal conditions for maximizing PPO activity were similar for all four cultivars: a pH of 6.0, a temperature of 30°C, and an enzyme concentration of 1% (v/v). For all cultivars, PPO activity decreased with increasing inactivation time after the temperature reached 30°C. The study tested the inhibitory effects of compounds such as ascorbic acid, citric acid, sodium chloride, sodium metabisulfite, and EDTA on the activity of residual enzyme. Ascorbic acid and sodium metabisulfite were the most effective inhibitors for these eggplant PPOs.***

**Keywords:** Eggplant, Polyphenol oxidase, Browning, *Solanum melogena*, Inhibitors

## INTRODUCTION

Browning in fruits and vegetables is considered undesirable. It shortens the shelf-life of fresh-cut fruits and vegetables by reducing their visual appearance, and in fresh products, it is associated with a loss of nutritional value (McEvily et al., 1992). Browning is caused by enzymatic oxidation of natural phenolic compounds in fruit tissue in the presence of oxygen. The oxidation of phenolic substances to quinones, catalyzed by polyphenol oxidase, is the primary cause. The quinones then condense to form dark pigments (Beaulieu et al., 1999). Most current research

focuses on controlling enzymatic browning in fruits and vegetables. However, browning substances from plants benefit the food industry in the processing of black tea (Ullah, 1991), coffee (Amorim, 1991), and cocoa (Lee et al., 1991); and as possible natural coloring agents. The intensity of the brownness depends on two important factors, the phenol content and the activity of PPO in the plant tissue.

Several cultivars of eggplants are consumed as raw and cooked vegetables in Thailand. The common edible eggplants are mainly from the cultivars of *S. melogena,* with a vast variety of peel colors and fruit shapes. These eggplants can be divided into two groups according to their morphological and cytological characteristics (Panpeng, 2003). The four eggplant cultivars used in this study can be divided into the two groups according to their texture. RG has a firm fruit texture and numerous seeds, while OG, OW, and OP have a tender to spongy fruit texture and scarcely any seeds. However, all of the fruits in this study brown differently. Browning of eggplants develops on the seeds, pulp, and skin of the fruit when they are prepared for cooking or processing. Browning occurs as the result of the high phenol content in the fruit (Whitaker and Stommel, 2003) and the high activity of PPO in their tissue (Fujita and Tono, 1988; Perez-Gilabert and Garcia-Carmona, 2000). PPO from *S. melogena* fruits have been partially purified and characterized (Perez-Gilabert and Garcia-Carmona, 2000; Dogan et al., 2002; Concellon et al., 2004; Cheriot et al., 2006; Zhang and Chen, 2006) but research is limited comparing the characteristics of PPO obtained from different eggplant cultivars (Zhang et al., 2006) that have different fruit characteristics and peel colors. The present study characterized the PPO from four cultivars of eggplant of *S. melongena*, with different fruit characteristics, textures, and peel colors, and studied the effects of substrate specificity, temperature, pH, thermal stability, and inhibitors on PPO activity to determine the most appropriate optimal condition for generating a natural browning agent from eggplants.

## MATERIALS AND METHODS

### Enzyme extraction

Four cultivars of eggplants with different sizes and peel colors were purchased from local markets and stored at 8°C. They included medium round green fruit (RG), small and long purple fruit (OP), elongated ovate white fruit (OW), and elongated ovate green fruit (OG), all of which were at commercial maturity. The extraction procedure was modified from Concellon et al. (2004). Eggplants were cut into small pieces and immediately frozen in liquid nitrogen. The frozen samples were ground to a fine powder and then freeze dried (Flexi-DryTm MP, Kinetics) at -70°C. Five replicates of 50 mg freeze-dried samples were extracted in 1.5 ml of phosphate buffer (0.1 M $KH_2PO_4$, 0.1 M $Na_2HPO_4$, pH 6.0), supplemented with 30 g/l of polyvinyl-polypyrrolidone (PVPP). The suspension was shaken on ice for 30 minutes and centrifuged at 20,000g (Zentrifugen Mikro 22R, Heltich) for 20 minutes at 4°C. Aliquots of the supernatant containing PPO were taken and stored at -80°C for further assays.

**Enzyme assays**

PPO activities from the extractions were carried out in standard microtitre plates, using a protocol according to Concellon et al. (2004) with some modification of the assay. Unless otherwise stated, the reaction mixture (200µL) was composed of 10 mM 4-methylcatechol in phosphate buffer (0.1 M $KH_2PO_4$, 0.1 M $Na_2HPO_4$, pH 6.0) and 50µL of enzyme extract (1% v/v). Blank references were prepared by mixing the substrate with the boiled enzyme extract. Changes in absorbance at 410 nm were recorded every 10 seconds for three minutes (Spectra MR, Dynex Technologies). The reactions were carried out at 30°C in triplicate measurements.

**Protein determination**

Protein content of the different extracts was measured according to the dye binding method (Bradford, 1976), with slight modification for the microplate reader determination, using bovine serum albumin (BSA) as the standard protein.

**Evaluation of enzyme properties**

**Substrate specificity and substrate concentration.** The activities of PPO from each eggplant cultivar were tested, using the extract and five substrates: 4-methylcatechol, 4-*tert* butylcatechol, catechol, dopamine, and catechin. All of the substrates were obtained from Sigma-Aldrich Chemical Co. (Singapore). The reaction mixture and the enzymatic activity for each cultivar were determined at 30°C, pH 6, and 1% (v/v) enzyme (standard condition). The enzyme extracts were mixed with several substrate concentrations, in the range of 5-20 mM. The enzymatic activity under each substrate reaction and for each cultivar was expressed in relative form as the percentage of the highest activity of PPO for that cultivar when using 4-methylcatechol as a substrate.

**Enzyme concentration.** The enzymatic activity of each cultivar was studied over a range of extract concentrations from 1 to 20% (v/v). Fifty µL of each dilution was mixed with 150µL of substrate to produce the 200µL total volume for each assay. PPO activities were measured under the standard conditions as previously described. The measurements were carried out in triplicate.

**Optimum pH.** The enzymatic activity of each cultivar was also determined over a pH range of 5.0-8.0, by adjusting the pH of 0.1 M phosphate buffer, using 10 mM 4-methylcatechol as a substrate. Enzymatic activities of each cultivar were determined according to the procedure as previously described. All assays were performed in triplicate.

**Optimum temperature.** PPO activities of the enzyme extracts from the different cultivars were studied over a temperature range of 2-80°C. The effect of temperature on the activities of different PPOs was tested by heating the phosphate buffer to an appropriate temperature, then mixing with the substrate before the

introduction of enzyme. Once the preference temperature was reached, the enzyme was added (Arslan et al., 2004) and the assay was immediately determined at the constant temperature. All assays were performed in triplicate.

**Thermal stability.** Diluted enzyme solutions (1% v/v) in phosphate buffer pH 6.0 from the extracts of each cultivar were incubated at 30, 40, 50, and 60°C. The incubation periods for each temperature were 15, 30, 45, and 60 min. Residual PPO activity was measured under standard assay conditions. The measurements were carried out in triplicate.

**Inhibitors.** The inhibitors examined included ascorbic acid, citric acid, EDTA, sodium chloride (NaCl), and sodium metabisulfite. To determine the effect of inhibitors, the standard reaction medium was modified slightly to maintain the final concentration of the substrate and inhibitors. Concentrations of the inhibitors were varied; in the range of 0.5-2.5 mM for citric acid, NaCl, and sodium metabisulfite and in the range of 2-20 mM for EDTA and ascorbic acid. The effect of inhibitors on the activity of PPO was tested by mixing the enzyme with an appropriate concentration of inhibitors before introducing the substrate. The substrate solution was added before spectrophotometric measurement. All assays were performed in triplicate.

**Statistical analysis**

Values are the average of three independent determinations. Cultivar effects were examined by analysis of variance (ANOVA) and mean differences were determined with the LSD test at the 0.05 level of significance, using the Microsoft Excel data analysis software package (Microsoft® Office Excel 2003).

## RESULTS AND DISCUSSION

**PPO extraction and specific activity**

PPO in plants is found in subcellular locations of the intact cell, mostly in plastids or chloroplasts. PPO preparations from eggplant fruits were found in both soluble and insoluble forms, but the enzymatic activity of soluble PPO was twice as high as the insoluble PPO (Concellon et al., 2004). The activity of PPO from eggplant exhibits both monophenolase and diphenolase activities (Perez-Gilabert and Garcia-Carmona, 2000), with diphenolase or catecholase activity predominating monophenolase activity.

In the present study, PPOs from eggplants of four cultivars were prepared from freeze-dried samples in phosphate buffer to obtain the soluble protein as described by Concellon et al. (2004). The amount obtained suggested that most of the eggplant PPO is not membrane-associated. The catecholase activity was determined from the soluble protein under the conditions as described in the standard assay condition. Each cultivar PPO showed different specific activity (Table 1) when measured with 10 mM 4-methylcatechol as substrate. Under this

condition, the values of Km and Vmax obtained from all cultivars; RG ($K_m$: 2.9 mM, $V_{max}$: 0.15 ΔOD $min^{-1}$), OG ($K_m$: 3.2 mM, $V_{max}$: 0.10 ΔOD $min^{-1}$), OW ($K_m$: 2.9 mM, $V_{max}$: 0.14 ΔOD $min^{-1}$), and OP ($K_m$: 2.8 mM, $V_{max}$: 0.20 ΔOD $min^{-1}$) were similar to previous reports of eggplant PPO (Perez-Gilabert and Garcia-Carmona, 2000; Dogan et al., 2002; Concellon et al., 2004).

**Table 1.** Eggplant cultivar characteristics, protein contents, and specific PPO activity.

| Cultivars | Fruit character and peel color | Fruit weight (g FW) | Protein content (mg gDW-1)[a] | Specific activity (ΔOD $min^{-1}$ $mgProtein^{-1}$[b]) |
|---|---|---|---|---|
| Choa Pra Ya (RG) | Round to ovate, uniform green | 23.8 ± 2.1 | 13.6 ± 0.2 | 305.29 ± 16.56 |
| Khaew yoa (OG) | Long ovate, uniform green | 81.9 ± 4.5 | 29.5 ± 0.3 | 562.89 ± 26.11 |
| Khaow yoa (OW) | Long ovate, uniform white | 83.0 ± 6.2 | 18.4 ± 0.3 | 631.33 ± 38.29 |
| Moung khan khew (OP) | Long linear to ovate, mottle purple | 18.5 ± 1.6 | 18.8 ± 0.1 | 652.54 ± 9.59 |

Note: [a]Protein content $gDW^{-1}$ = total soluble protein per gram of dry weight. [b]unit mgProtein-1 = one unit of PPO activity was defined as the amount of the enzyme from one milligram of soluble protein that causes a change in absorbance of 0.1 in one minute under standard assay with 10 mM 4-methylcatechol, in 0.1M phosphate buffer pH6.0, at 30°C.

**Physiochemical properties of PPO**

**Substrate specificity and substrate concentration.** PPO is active to phenolic compounds that have a high preference to the enzyme. The structure of the compound, type of side chain, number of hydroxyl groups, and their position in the benzene ring have a major effect on the catalytic activity of the enzyme (Yoruk and Marshall, 2003). In the present study, PPO from different eggplant cultivars had varying substrate specificities at different concentrations (Figure 1). The PPO from RG oxidized 4-methylcatechol at all concentrations at a much faster rate than other structurally-related substances, while the activity of PPO from OG was the greatest with 4-*tert* butylcatechol, followed by catechin and 4-methylcatechol. The substrate with the highest activity for OW and OP was catechin, followed by 4-methylcatechol and 4-*tert* butylcatechol. Catechol, 4-methylcatechol and 4-*tert* butylcatechol have previously been reported to be the specific substrates for PPO from other varieties of eggplants with different concentrations (Perez-Gilabert and Garcia-Carmona, 2000; Dogan et al., 2002; Concellon et al., 2004; Cheriot et al., 2006; Zhang et al., 2006). Catechin is a major phenolic compound found in grapes (Jaworski and Lee, 1987) and tea (Ullah, 1991) and is believed to be a common natural substrate of several other fruit PPOs. This substrate has not been used as the substrate for eggplant PPO before. Its structure, thus possible stronger substrate affinity, explains the higher activity for PPO of OW and OP.

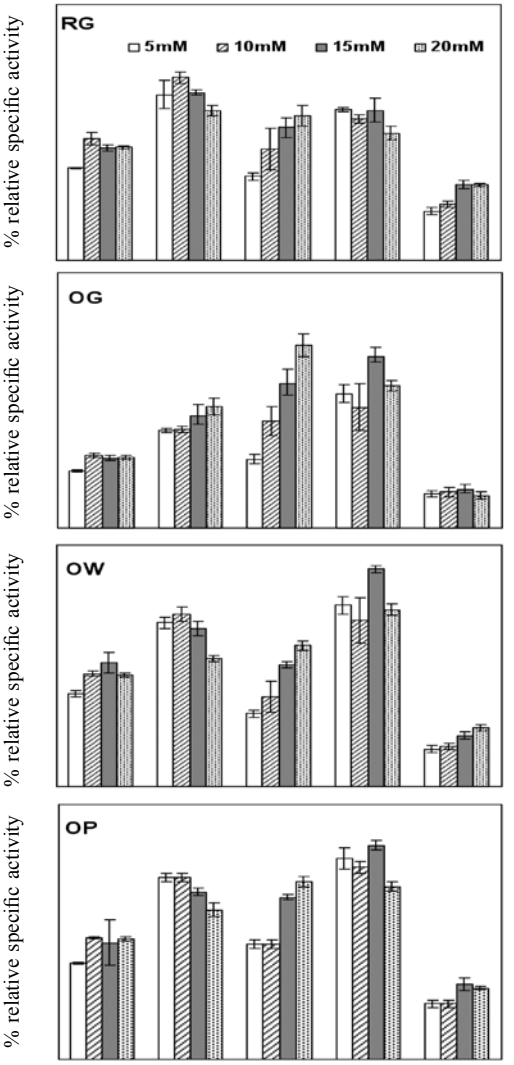


**Figure 1.** Substrate specificity and concentration for PPOs from each cultivar of eggplant.

Note: Catechol (CAL), 4-methylcatechol (4-MC), 4-tertbutylcatechol (TBC), catechin (CAT), and dopamine (DOP). Values are means of triplicate determinations ± SE.

PPO from eggplants also showed activity toward dopamine but not as high as the activity reached by the other substrates. It appears that substrate specificity of eggplant PPO is dependent on cultivars, as was found in a previous report (Dogan et al., 2002). However, $K_m$ and $V_{max}$ values of the enzyme-catalyzed reaction with catechin; RG ($K_m$: 2.7 mM, $V_{max}$: 0.20 **Δ**OD min-1), OG ($K_m$: 3.9 mM, $V_{max}$: 0.09 **Δ**OD $min^{-1}$), OW ($K_m$: 2.7 mM, $V_{max}$: 0.14 **Δ**OD $min^{-1}$) and OP ($K_m$: 2.9 mM, $V_{max}$: 0.22 **Δ**OD $min^{-1}$) were not comparable to those of the $K_m$ and $V_{max}$ values with 4-methylcatechol as substrate. Therefore, 4-methylcatechol with the concentration of 10 mM was used as the substrate for further standard assay.

**Effect of enzyme concentration.** The specific activities of eggplant PPO, using 4-methylcatechol as substrate, showed a linear increase with the enzyme concentration (Figure 2). PPO activity from each cultivar reached a steady state at different concentrations. For further experiments, the enzyme concentration was set to 1% (v/v), corresponding to the linear portion of the curve for all four cultivars.

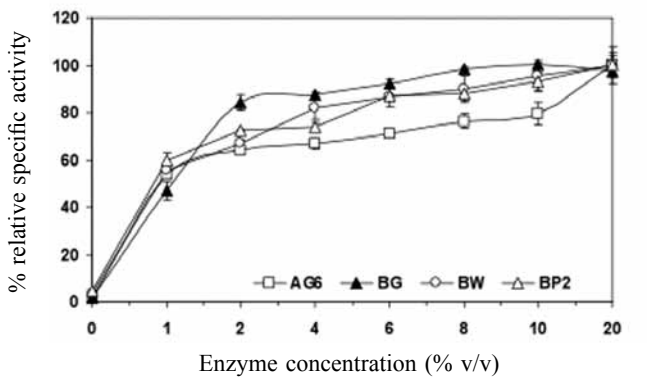


**Figure 2.** Effect of enzyme concentration on the specific activities of PPOs.
Note: Values are means of the relative activity expressed as a percentage of the maximum activity of each concentration ± SE.

**Effect of pH.** Different pH optima were determined for PPO from each cultivar, using 4-methylcatechol as a substrate. Results are presented in Figure 3. These enzymes showed similar profiles of PPO activity. PPOs from all cultivars were active between pH 5 to 8. They had a broad optimum between 5.0 and 6.5 and then continuously declined. Eggplant PPO has a wide range of optimum pH (4.8-6.0) with 4-methylcatechol as substrate. Catecholase activity showed a broad maximum activity at pH 5.0-5-5 (Perez-Gilabert and Garcia-Carmona, 2000). The activity of PPO from other cultivar rapidly decreased below pH 4.8 and slightly decreased from pH 6.0 (Concellon et al., 2004).

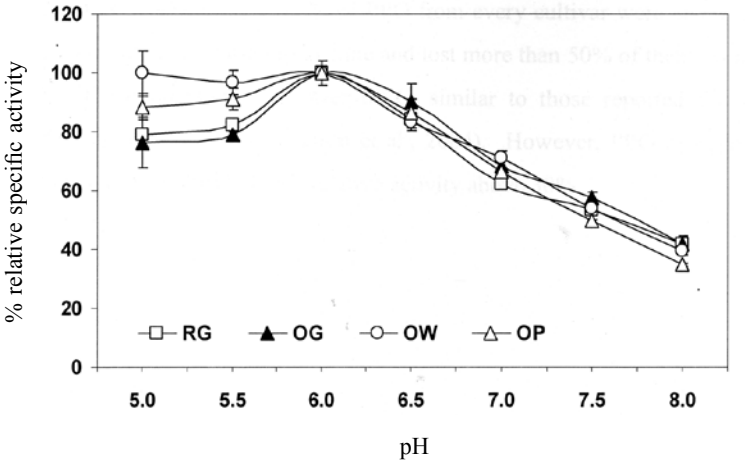


**Figure 3.** Effect of pH on the specific activities of PPOs.

Note: Values are means of triplicate determinations ± SE represented as the relative activity expressed as a percentage from the highest PPO activity reached for each cultivar.

**Effect of temperature.** Temperature is another important factor that affects the enzymatic activity of PPO. The optimum temperature of PPO varies for different plant sources (Yoruk and Marshall, 2003). PPO from each cultivar of eggplant was determined between 2 and 80°C. Figure 4 shows the effect of temperature on the PPO activity when 4-methylcatechol was used as the substrate. Optimum temperatures for maximum PPO activity were 30°C for RG, OG, and OW, then decreased gradually with increasing temperatures. PPO from OP showed fluctuations in activity between 2 and 30°C. The optimum temperatures for PPO from OP cultivars were 10 and 30°C ($P>0.05$). The relative activities at the temperature optima were considered as 100% of the specific activity. The relative specific activities between 2 and 40°C of PPO from every cultivar were above 80%. Their enzymatic activities started to decline and lost more than 50% of their oxidizing ability when the temperature exceeded 60°C, similar to those reported for other varieties (Dogan et al., 2002; Concellon et al., 2004). However, PPO of eggplants remained active even at 80°C, with a relative activity above 30%.

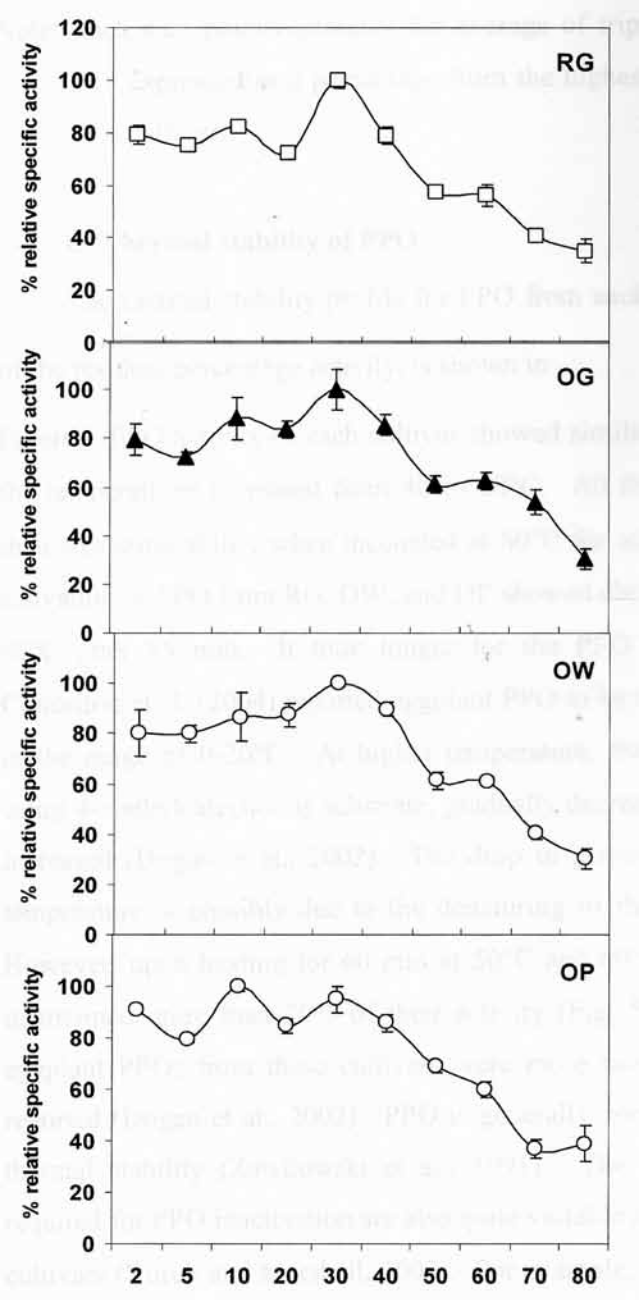


**Figure 4.** Optimal temperature for the specific activities of PPOs.

Note: Each data point represents the average of triplicate determinations ± SE and expressed as a percentage from the highest PPO activity reached for each cultivar.

**Thermal stability of PPO.** The thermal stability profile for PPO from each cultivar, presented in the form of the residual percentage activity, is shown in Figure 5. PPO activity of each cultivar showed similar profiles of thermal stability as the temperatures increased from 40 to 60°C. All the PPOs lost more than 50% of their oxidizing ability when incubated at 60°C for at least 15 min. For instance, the activation of PPO from RG, OW, and OP showed about a 50% reduction in activity at 60°C after 15 min. It took longer for the PPO from OG to become inactive. Concellon et al. (2004) reported eggplant PPO to be thermostable at low temperature, in the range of 0-20°C. At higher temperature, the time required for inactivation, using 4-methylcatechol as substrate, gradually decreased as the time and temperature increased (Dogan et al., 2002). The drop in percentage residual activity at higher temperature is possibly due to the denaturing of the enzyme (Dogan et al., 2005). However, upon heating for 60 min at 50°C and 60°C, all the enzyme residuals still maintained more than 20% of their activity (Fig. 5). The results suggest that the eggplant PPOs from these cultivars were more heat tolerant than those previously reported (Dogan et al., 2002). PPO is generally considered to be an enzyme of low thermal stability (Zawitowski et al., 1991). The exposure time and temperature required for PPO inactivation are also quite variable among different plant species and cultivars (Yoruk and Marshall, 2003). For example, heat treatment up to 50°C for 20 min could reduce the activity of PPO from longan by 50% (Jiang et al., 1999), whereas the activity of lettuce PPO did not show any reduction after being treated at 70°C for 5 min (Heimdal et al., 1994).

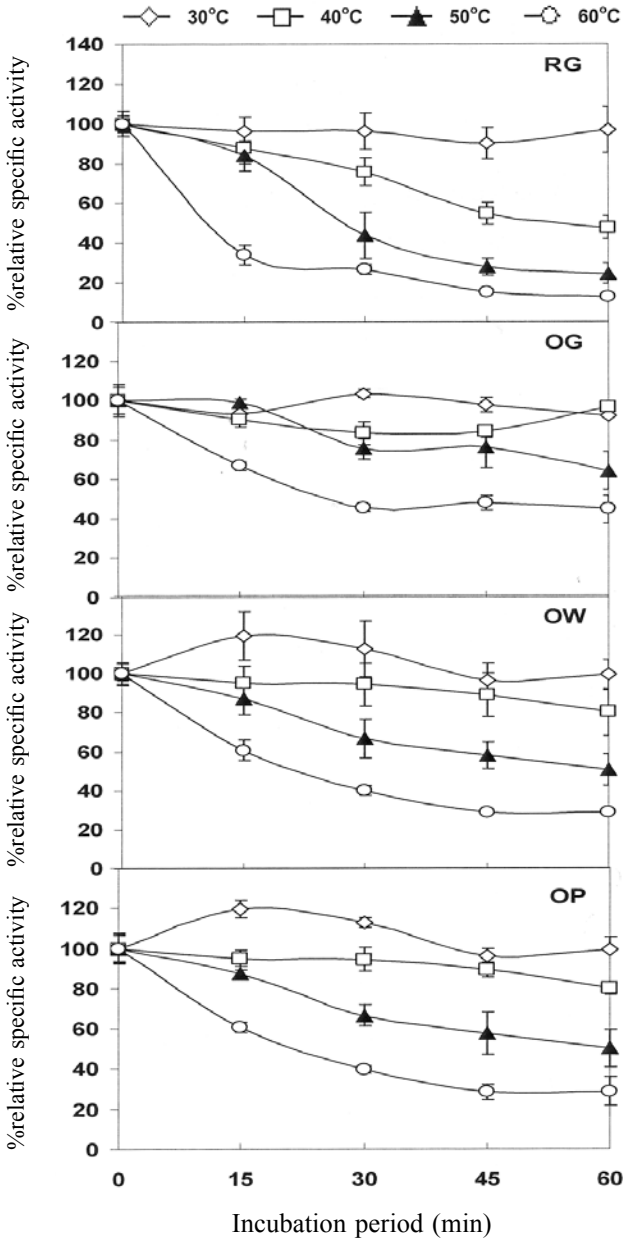


**Figure 5.** Thermal stability of PPOs with respect to incubation time at different temperatures.

Note: Values are means of triplicate determinations ± SE and expressed as a percentage of the initial activity.

**Inhibitors.** Various inhibitors were examined to determine their potential for inhibition of eggplant PPO activity from each cultivar. These inhibitors included reducing agents (citric acid, sodium metabisulfite), a chelating agent (EDTA), and an acidulant (citric acid). These compounds diminish or inhibit the browning reaction rate by means of eliminating enzyme, substrate, copper, or a reaction intermediate from the reaction. Furthermore, PPO from different sources may react similarly with inhibitor compounds, but the effectiveness of inhibitors against the PPOs could vary (Ferrar and Walker, 1996). As seen, the most effective inhibitors for PPO for all cultivars were ascorbic acid and sodium metabisulfite, at the lowest concentration tested (Fig. 6). However, the sensitivity of PPO to EDTA and NaCl was different from cultivar to cultivar. Activity of PPO from OG was inhibited more than 50% when the concentration of NaCl increased to 1.0 mM, while it had little effect on the enzymatic activity of PPO from RG, OW, and OP, which showed 28%, 12%, and 33% inhibition, respectively. Adiculants are widely used in food processing to control browning, given acids (such as ascorbic, citric, and malic acids) are naturally present in some edible food products. Citric acid also functions as a PPO inhibitor through its chelating action (Eskin et al., 1971). Citric acid only slightly affected the activity of eggplant PPO as the amount of acid may not be enough to compete with the enzyme-substrate reaction. PPO activity of water chestnut was inhibited when the concentration of citric acid was 0.1M or higher (Jiang et al., 2004). Normally, citric acid is used along with another inhibitor for better control of PPO-induced browning, such as in combination with ascorbic acid (Eskin et al., 1971) or glutathione (Jiang and Fu, 1998). The result suggested that ascorbic acid and sodium metabisulfite were the most effective inhibitors of eggplant PPO. Sodium metabisulfite is banned for use with fresh fruits and vegetables due to safety concerns (Martinez and Whitaker, 1995). A combination of ascorbic acid, citric acid, and heat treatment was proposed as an alternative method to control the PPO of eggplant (Almeida and Nogueira, 1995).

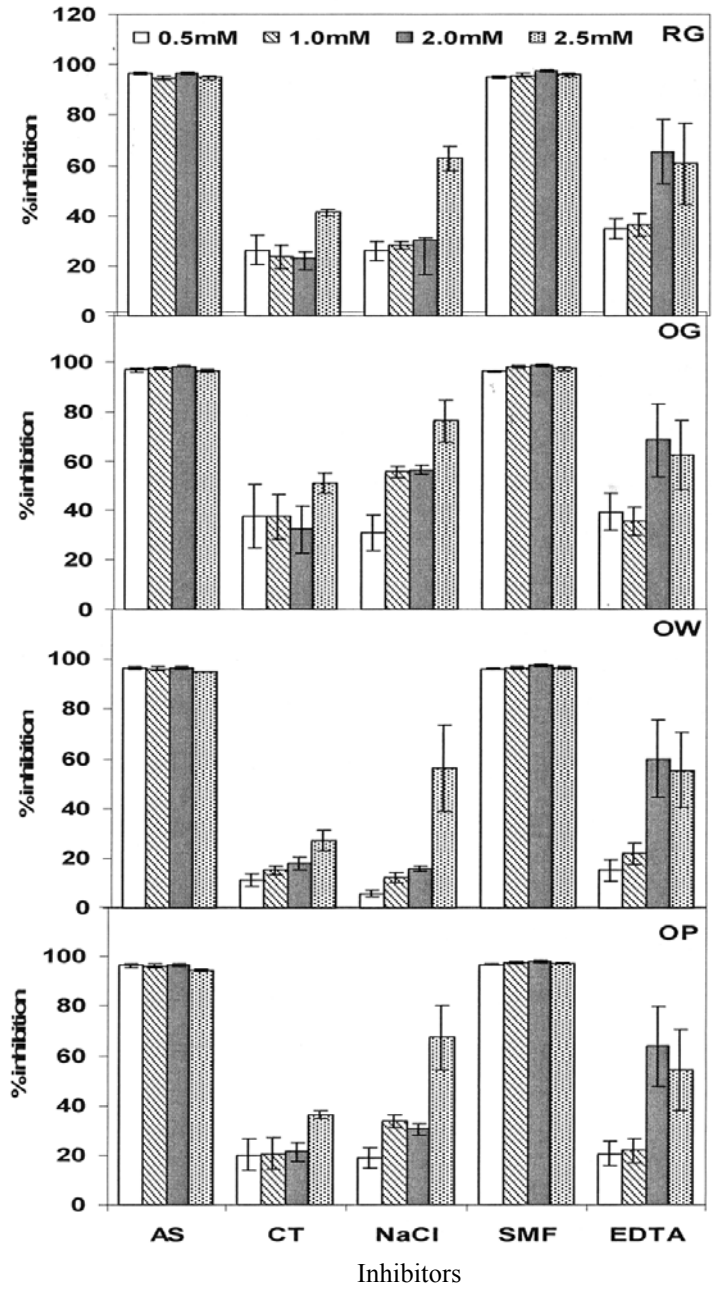


**Figure 6.** Effect of inhibitors on the residual enzymatic activity of PPO from different cultivars of eggplant fruits.

Note: Inhibitors were AS (ascorbic acid), CT (citric acid), NaCl (sodium chloride), SMF (sodium metabisulphite) and EDTA. Values are mean of triplicate determinations ± SE and represent as a percentage of inhibitory effect on the PPO activity.

## CONCLUSION

Fruit texture and peel color did not influence the enzymatic activity of eggplant PPO. The PPO had different substrate preferences but the conditions for enzyme activity were similar. The most effective substrate was 4-methylcatechol for PPO from RG, 4-*tert* butylcatechol for PPO from OG, and catechin for PPO from OW and OP. The optimal conditions for these PPO to perform their browning reaction were a pH of 6.0, a temperature of 30°C, a 1% (v/v) enzyme concentration, and a substrate of 10 mM 4-methylcatechol. PPO from all cultivars showed thermal stable properties and had broad optimal temperatures (2-40°C). Nevertheless, their activity decreased with an increase in temperature and inactivation time. Ascorbic acid and sodium metabisulfite were the most effective inhibitors for the eggplant PPOs used in this study. These compounds should be avoided if the maximum PPO enzymatic reaction is desired. PPO from the eggplant cultivars studied here are a potential source for natural browning agents for the food industry if used under the above optimal conditions.

## ACKNOWLEDGEMENTS

This research was financed by the Thailand Research Fund (TRF) and supported by the Postharvest Technology Research Institute and Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

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