Screening Methods to Measure Antioxidant Activities of Phenolic Compound Extracts from Some Varieties of Thai Eggplants

U. Chanasut
Postharvest Technology Institute
Chiang Mai University
Chiang Mai, 502000
Thailand

N. Rattanapanone
Faculty of Agro-Industry
Chiang Mai University
Chiang Mai, 50100
Thailand

Keywords: *Solanum melogena*, *Solanum torvum*, DPPH, β-carotenoid bleaching, $ABTS^+$

Abstract

Phenolic compounds and their antioxidant activities from fruits of eleven Thai eggplant varieties were determined. Freeze dried samples were extracted in 70% methanol and their total phenolic contents were measured. Antioxidant activities of the crude extracts were evaluated using three different assays: the DPPH (diphenyl-1-picrylhydrazyl) assay, the β-carotenoid bleaching assay, and the $ABTS^+$ (2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) assay. The antioxidant activities were expressed as gallic acid equivalents (GAE) to standardize these methods to allow for data comparisons. The three antioxidant assay methods give different antioxidant activity trends. Phenol contents of the eggplant extracts were related to their antioxidant activities measured by $ABTS^+$ and DPPH assay. In contrast, the results measured by the β-carotenoid bleaching assay were not related to the phenol contents of the extracts. On the basis of DPPH results, *Solanum torvum* has both the highest total phenolic content and total antioxidant activity.

INTRODUCTION

Eggplants are of considerable importance in the Thai diet. They are consumed as vegetables and use as ingredients in Thai cooking. Some eggplant species exhibit medicinal properties and are used as such. There are at least 20 species of Thai eggplants. The common edible eggplants in Thailand are *S. melogena*, *S. nigrum*, *S. torvum* and *S. wendlandii* (Panpeng, 2003). Thai eggplant fruit exhibits diversity in shape, size and color, depending on the cultivar. They are also rich with phenolic compounds, especially the browning related substances. Phenolic compounds are very effective free radical scavengers; therefore, they are a potential source of antioxidants. The eggplant is ranked among the top ten vegetables in term of antioxidant capacity (Coa et al., 1996). This is attributed to the phenolic constituents in the fruit. Fourteen phenolic compounds in eggplants have been previously characterized and identified (Stommel and Whitaker, 2003). Plant phenolic compounds have been ascribed to promoting several health effects. It has been proposed that consuming phenolic-compound rich fruits and vegetables may reduce several aging-related chronic diseases including cancer and heart disease (Halliwell et al., 1992). Therefore, eggplants could thus be promoted as an important source for functional food and other applications for Thai people. However, data on eggplant phenolic compounds and antioxidant activities are mainly focused on *S. melogena* (Hansona et al., 2006; Stommel and Whitaker, 2003). Such information is needed if eggplants are to become a source of the phytochemicals for Thai consumers. A quick, reliable method for evaluating antioxidant activities from eggplant fruit samples from various species and cultivars is necessary.

The phenolic content of foods and biological systems is commonly expressed as an amount of gallic acid equivalents. It is therefore important to consider the type of standard used to express the antioxidant activity when testing the antioxidant properties of phenolic compounds in vitro. Several methods are used to determine antioxidant activities of natural compounds in food. Two free radicals commonly used to evaluate antioxidant activities in vitro are $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. The ABTS assay measures the relative ability of antioxidants to scavenge the $ABTS^{\bullet+}$ generated in the aqueous phase and compared

Proc. XXVII IHC - Plants as Food and Medicine
Eds.-in-Chief: G. Gardner and L.E. Craker
Acta Hort. 765, ISHS 2008

with a soluble vitamin E analogue, Trolox (Miller and Rice-Evan, 1997). The DPPH is a stable free radical with an absorption wavelength at 515 nm. Its absorbance is reduced by an antioxidant, and the reduction in absorbance can be measured with a spectrophotometer (Sánchez-Moreno, 2002). Gallic acid was used as a standard in this assay. The β-carotene bleaching assay is used to test antioxidants in foods and biological systems and consists of oxidizing a lipid or lipoprotein substrate under standard conditions. This assay measures the inhibition capacity of antioxidants in protecting the bleaching of β-carotene, emulsified with linoleic acid, by the free radical (Hammerschmidt and Pratt, 1978) compared with a synthetic antioxidant, butylated hydroxytoluene (BHT).

It is interesting to evaluate these antioxidant testing methods using gallic acid as a standard. These methods will be standardized in order to estimate and compare the antioxidant activities of eggplant phenolic compounds. In the present study, the total phenolic contents from 11 cultivars of Thai eggplants were measured and their antioxidant activities were evaluated using the ABTS assay, the DDPH assay and the β-carotene bleaching assay with gallic acid as a standard. The correlation between total phenolic content and total antioxidant activities was determined.

MATERIALS AND METHODS

Plant Materials

Eleven cultivars of eggplant obtained from local markets in Amphur Meung, Chiang Mai, were used in this study. The fruit were immediately frozen in liquid nitrogen after cutting and then vacuum dried. The freeze-dried samples were stored in air tight desiccators for further analyses.

Methods

Freeze dried samples were ground into fine powder. Five replicates of 50 mg sample powder were extracted for an hour in 70% methanol, then centrifuged at 10,000 g for 15 minutes at room temperature, and aliquots were kept at -70°C for subsequent analyses of total phenolic content (TPC) and total antioxidant activity (TAA).

TPC was measured by the colorimetric method with Folin-Ciocalteu reagent (Keta and Atantee, 1998) and gallic acid was used as a standard. Total phenols were expressed as mg gallic acid equivalent per gram dry weight (mg GAE). TAA was estimated by measuring the relative ability of antioxidants to scavenge the $ABTS^{\bullet+}$ (Nenadis et al., 2004) and $DPPH^{\bullet}$ (Sanchez-Moreno et al., 1998). The ability to inhibit the bleaching of the carotenelinoleate solution was also measured (Dapkevicius et al., 1998). These colorimetric methods were slightly modified and the assay volumes were adjusted in order to measure in a spectroscopic microplate reader (Dynex Technologies, Spectra MR). Gallic acid was used as a standard and TAAs were expressed as mgGAE per gram dry weight.

Data were reported as mean ± standard error of mean (SE) for five replicate determinations (Microsoft Excel© XP version, Microsoft Corp.). Pearson correlation test was conducted to determine the correlations among means.

RESULTS

Total Phenolic Content

The eggplant fruit samples were *S. melongena*, *S. torvum* and *S. nigrum* (Table 1). The samples were examined and compared for their TPC expressed as mg GAE (Fig. 1). The eleven eggplant samples differed from one another in their TPC. The greatest TPC of 47.5 mg GAE/g dry weight was detected in the *S. torvum* (AG4) whereas the *S. melogena* (AG2) has the lowest TPC value of 11.2 mg GAE/g dry weight.

Total Antioxidant Activity

1. ABTS assay. Phenolic extracts of the eleven eggplant fruit samples were examined and compared for their free radical scavenging activities against the radical cation $ABTS^{\bullet+}$. All extracts showed $ABTS^{\bullet+}$ scavenging capacity (Fig. 2). The greatest antioxidant capacity was detected in phenolic compounds extracted from *S. torvum* (AG4), followed by *S. melogena* (AG3). The scavenging capacities of the phenolic compounds were also correlated to the TPC of the fruits ($r^2 = 0.76$), especially the green color peel (AG group). AG4 had the greatest both phenolic content and scavenging capacity according to the radical cation $ABTS^{\bullet+}$ scavenging activity.

2. DPPH assay. The methanol extracts were analyzed and compared with gallic acid as a standard for antioxidant activity. The entire phenolic compounds extracted from eggplants were capable of directly reacting with and quenching $DPPH^{\bullet}$ (Fig. 2). Furthermore, the antioxidant activities were greater than that determined by the ABTS assay. The greatest amount of reduction of DPPH was detected in phenolic compounds extracted from AG4, which had the highest content of phenolic compounds. The TAA was also correlated ($r^2 = 0.80$) to the TPC of the green eggplants (Fig. 3).

3. β-Carotene–Linoleic Acid Assay. The antioxidant activities of eggplant fruit extracts measured by the bleaching of β-carotene are presented in Fig. 2. This assay gave different antioxidant activity trends compared to the DPPH and ABTS assays. Phenolic compounds extracted from *S. melongena* (AG6) were found to give the maximum antioxidant activity. The lowest antioxidant activity was the eggplant methanol extracts from AG4. The result also indicated that the antioxidant activity examined by this assay was not correlated with the phenolic content of eggplants (Fig. 3).

DISCUSSION

Previous studies have reported that phenolic extracts from *S. melongena* fruit with purple peels has high antioxidant activity and ranked in the top ten antioxidant-rich vegetables (Coa et al., 1996; Keneyuki et al., 1999). Anthocyanins in the eggplant peels may play an important role in those studies. However, in results of our study indicated that green peel eggplants had higher phenolic content and also greater antioxidant activity estimated than that of those with purple peels by the ABTS and DPPH assays. The green peel eggplant, for example, *S. torvum*, a tiny dark green fruit, is an important ingredient in Thai cooking. Thai people usually eat green peel eggplant (*S. melogena*) fruit, except BG1, as a fresh vegetable for salads and dips. Thus, these eggplants are already a major part of the Thai diet. To achieve the maximum benefits from the eggplants as a functional food, it is necessary to understand the distribution of phenolic compounds and their ability as antioxidants.

The TAA estimated from three assays gave varied results ranging from 1.2 to 18.0 mg GAE of dry material. The ABTS and DPPH assay gave similar TAA results, which were correlated to the TPC of eggplant extracts. Although the β-carotene bleaching assay is commonly used to evaluate the ability of plant phenol extracts to prevent the oxidation of the β-carotene/linoleic acid, our study showed that the TPC and TAA of eggplant extracts were not correlated. The ABTS assay is operationally simple, and it is widely used in a variety of research for studying antioxidant activity of many compounds and food samples (Huang et al., 2005). However, the value is expressed as Trolox equivalent antioxidant capacity (TEAC). The DPPH assay is also widely used to determine antioxidant activity of natural plant extracts such as phenolic compounds. The benefit of this assay is that the antioxidant is expressed as mg of gallic acid equivalent (GAE). Gallic acid is a standard for the phenolic determination assay (Singleton and Rossi, 1965); therefore, it is a straightforward way to correlate the total phenolic content and antioxidant activity. The DPPH assay has good repeatability but color interference of samples that contain anthocyanins may occur and give an underestimation of antioxidant activity (Arnao, 2000). In this study, there were three samples of purple peel eggplants. Although the TAA results were lower compared to that of the green peeled eggplants, the color of methanol extracts of whole fruit samples was not different. Still, the TAA results

estimated from DPPH assay was greater than that of the ABTS assay expressed as GAE and more related to the TPC. Based on the DPPH results, *S. torvum* has both the greatest TAA and TPC. Therefore, the DPPH assay seem to be a better antioxidant measurement method to estimate the TAA of eggplant fruit phenolic extracts.

ACKNOWLEDGEMENTS

This work was financially supported by the Thailand Research Fund. We are also grateful to Citrus & Subtropical Laboratories and Research Center, USDA, Winter Haven, Florida and Postharvest Technology and Faculty of Science, Chiang Mai University for their support and other expenses.

Literature Cited

Arnao, M.B. 2000. Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. Trends Food Sci. Technol. 11:419–421.

Cao, G., Sofic, E. and Prior, R.L. 1996. Antioxidant capacity of tea and common vegetables. J. Agric. Food Chem. 44:3426–3431.

Dapkevicius, A., Venskutonis, R., van Beek, T.A. and Linssen, J.P.H. 1998. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. J. Sci. Food Agric. 77:140–146.

Halliwell, B., Gutteridge, J.M.C. and Cross, C.E. 1992. Free radicals, antioxidants, and human disease: where are we now? J. Lab. Clin. Med. 119:598–620.

Hammerschmidt, P.A. and Pratt, D.E. 1978. Phenolic antioxidants of dried soybeans. J. Food Sci. 43(2):556–559.

Hansona, P.M., Yanga, Ray-Yu, Tsoua, S.C.S., Ledesmaa, D. Englea, L. and Le, Tung-Ching 2006. Diversity in eggplant (*Solanum melongena*) for superoxide scavenging activity, total phenolics, and ascorbic acid. J. Food Com. Anal. 19:594–600.

Huang, D., Ou, B. and Prior, R.L. 2005. The Chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 53:1841–1856.

Kaneyuki, T., Noda, Y., Traber, M.G., Mori, A. and Packer, L. 1999. Superoxide anion and hydroxyl radical scavenging activities of vegetable extracts measured using electron spin resonance. Biochem. Molec. Bio. Inter. 47:979–989.

Ketsa, S. and. Atantee, S. 1998. Phenolic, lignin, peroxidase activity and increased firmness of damaged pericarp mangosteen fruit after impact. Postharvest Biol. Tech. 14:117–124.

Miller, N.J. and Rice-Evans, C.A. 1997. Factors influencing the antioxidant activity determined by the $ABTS^{+}$ radical cation assay. Free Radical Res. 26:195–199.

Nenadis, N., Wang, Lan-Fen, Tsimidou, M. and Zhang, Hong-Yu 2004. Estimation of scavenging activity of phenolic compounds using the $ABTS^{+}$ assay. J. Agric. Food Chem. 52:4669–4674.

Panpeng, B. 2003. Collection and phylogenetics of some *Solanum* spp. in Thailand. Postgraduate Thesis. Graduate School, Chiang Mai University (English abstract).

Sánchez-Moreno, C., Larrauri, J.A. and Saura-Calixto, F. 1998. A procedure to measure the antiradical efficiency of polyphenols. Sci. Food Agric. 76:270–276.

Sánchez-Moreno, C. 2002. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci. Technol. Int. 8(3):121–137.

Singleton, V.L. and Rossi, J.R. 1965. Colorimetry of total phenolics with phosphor-molybdic-phosphotungstic acid reagent. Amer. J. Enol. Vie. 16: 144 – 147.

Stommel J.R. and Whitaker B.D. 2003. Phenolic acid content and composition of eggplant fruit in a germplasm core subset. J. Am. Soc. Hort. Sci. 128:704–710.

Tables

Table 1. Species, peel colors, and fruit size of eggplant fruit materials used in the present study.

Sample no.	Species	Fruit color and size
AG1	*S. melogena*	Green strips, short round shaped, ϕ 2 cm (small)
AG2	*S. melogena*	Green strips, short round shaped, ϕ 6 cm (medium)
AG3	*S. melogena*	Green strips, short ovate shaped, ϕ < 3 cm (small)
AG4	*S. torvum*	Dark green, uniform round shaped, ϕ 1.5 cm (pea sized)
AG5	*S. nigrum*	Mottle green, short round shaped, ϕ < 1cm (small)
AG6	*S. melogena*	Pale green strips, round shaped, ϕ 5 cm (medium)
AW1	*S. melogena*	Uniform white, long linear shaped, ϕ 4 cm (large)
BG1	*S. melogena*	Uniform pale green, long linear shaped, ϕ 4 cm (large)
BP1	*S. melogena*	Purple strips, short ovate shaped, ϕ 4 cm (medium)
BP2	*S. melogena*	Mottle purple, long lanceolate shaped, ϕ 3 cm (medium)
BP3	*S. melogena*	Dark purple, oblong shaped, ϕ 8 cm (large)

ϕ = approximate diameter of the fruits

Figures

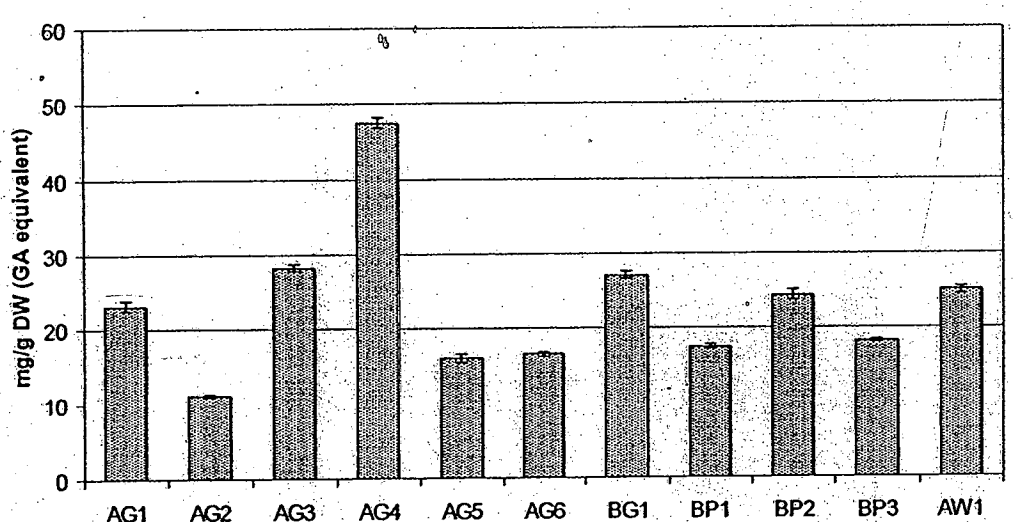


Fig. 1. Total phenolic content (TPC) of eggplant fruit extracts. Results are expressed as mg gallic acid equivalent. Values are means of five replicates ± SE.

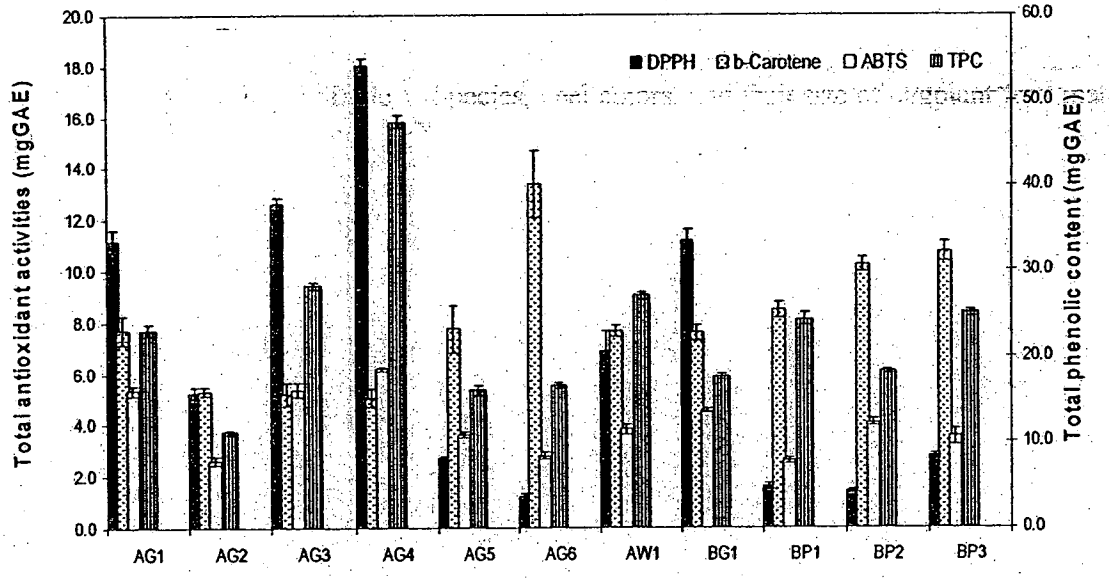


Fig. 2. Comparison of the antioxidant activities from the ABTS, DPPH and β-carotene bleaching assays and the TPC from methanol extracts from eggplant samples. The total antioxidant activity (TAA) and the TPC were expressed as mg gallic acid equivalent per gram dry weight. Values are means of five replicates ± SE.

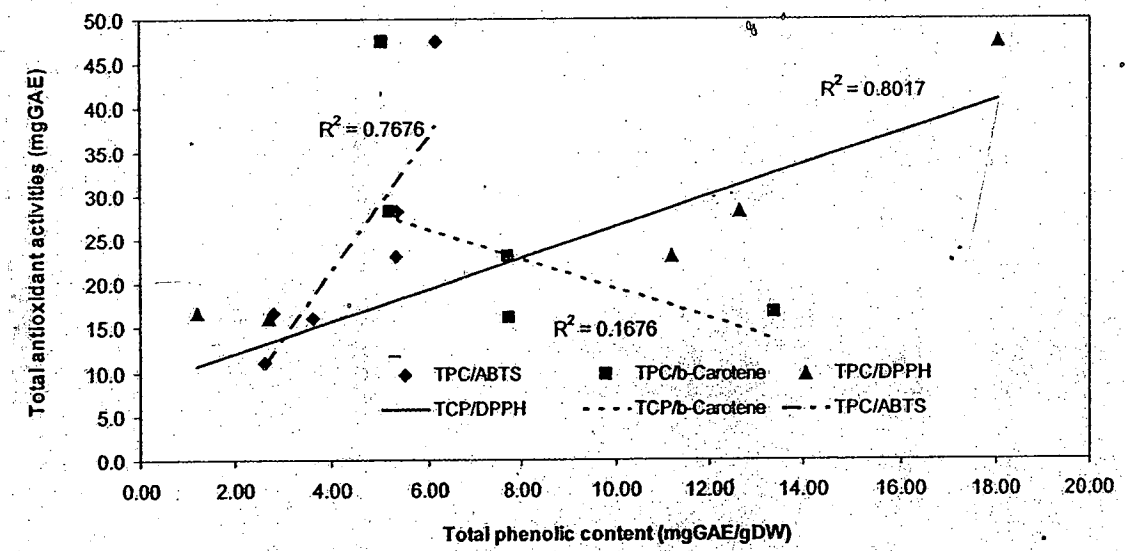


Fig. 3. Correlation between the TAAs from the ABTS, DPPH and β-carotene bleaching assays and the TPC of green peel eggplant samples. Values are expressed as mg gallic acid equivalent per gram dry weight basis.

Chiang Mai University Journal of Natural sciences
Office of the University, Chiang Mai University,Chiang Mai 50200, Thailand.

Phone: 66-53-94-2676-7 Fax: 66-53-94-3600
E-mail: cmujour@chiangmai.ac.th
Website: http://cmuj.chiangmai.ac.th,
or http://www.rac.oop.cmu.ac.th

Ref: No.6392(11)/662

June 7th, 2011

Ms. Usawadee Chanasut
Postharvest Technology Research Institute
Postharvest Technology Innovation Center
Chiang Mai University
Chiang Mai
50200

Dear Ms. Usawadee Chanasut:

Thank you for the manuscript entitled **"Characterization Comparative Study of Polyphenol Oxidases from Four Cultivar of Thai *Solanum melogena* Fruits"**, which you submitted for possible publication in the Chiang Mai University Journal of Natural Sciences.

I am pleased to inform you that the Editorial Committee has now agreed to accept your revised manuscript for publication.

Thank you for choosing the CMU journal of Natural Sciences as your preferred medium for publication.

Yours sincerely,

Chuckree Senthong

Professor Chuckree Senthong, Ph.D.
Editor-in-Chief
Tel: 053 94 2676
Fax: 053 94 3600
E-mail: cmujour@chiangmai.ac.th
http://cmuj.chiangmai.ac.th

Remark: The above article will be published in Chiang Mai University Journal of Natural Sciences Volume 11 Number 2 July – December, 2012.

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

Usawadee Chanasut*[1] and Nithiya Rattanapanone[1,2]

[1]*Postharvest Technology Research Institute / Postharvest Technology Innovation Center, Chiang Mai University, Chiang Mai 50200, Thailand*

[2]*Department of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, 50100, Thailand*

**Corresponding author. E-mail: chanasut@chiangmai.ac.th*

ABSTRACT

Polyphenol oxidases (PPO) from four cultivars of eggplant which have varietis of fruit characters, fruit textures and peel colors were characterized. The substrate specificity, temperature, optimal pH, thermal stability and inhibitors on the activities were compared. 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. However, the best substrate for PPO from each cultivar was different: for cultivars OW and OP, it was catechin, for Choa pra ya (RG), it was 4-MC and for Khaew yoa (OG), it was 4-tert butylcatechol. The optimal conditions for PPO to perform maximum activity from all cultivars were similar, using 4-methylcatechol as a substrate; they were pH 6.0, temperature of 30℃ and with 1% (v/v) enzyme concentration. The activities of all PPOs decreased with increasing temperature and inactivation time. The inhibitory effects of compounds such as ascorbic acid, citric acid, sodium chloride, sodium metabisulfite and EDTA on the activity of residual enzyme were tested. Ascorbic acid and sodium metabisulfite were found to be the most effective inhibitors for these eggplant PPOs.

Key words: Eggplant, Polyphenol oxidase, Browning, *Solanum melogena*, Inhibitors

INTRODUCTION

Browning in fruits and vegetables is considered undesirable: it shortens the shelf-life of fresh-cut fruit and vegetables by reducing their visual appearance, and in the fresh products, it is associated with loss of nutritional value (McEvily *et al.*,

1992). Browning is caused by enzymatic oxidation of natural phenolic compounds in the fruit tissue in the presence of oxygen. It occurs primarily by the oxidation of phenolic substances to quinines, catalyzed by polyphenol oxidase. The quinones then condense to form dark pigments (Beaulieu *et al.*, 1999). Most of the current research is focused on the control of enzymatic browning in fruit and vegetable. However, browning substances from plants also have a benefit in the food industry such as in the processing of black tea (Ullah, 1991), coffee (Amorim, 1991), cocoa (Lee *et al.*, 1991), and also as possible natural coloring agents. The intensity of the brownness depends on two important factors, the phenol contents 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* which has vast varieties of peel colors and fruit shapes. These eggplants can be divided into two groups according to their morphological and cytological characters (Panpeng, 2003). However, all of their fruits exhibit the browning in a different manner. Browning of eggplants develops on the seeds, pulp and skin of the fruit when they are prepared for cooking or processing. Their browning phenomenon occurs as the result of the high phenol contents 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 there is little research comparing the characteristic of PPO obtained from different eggplant cultivars (Zhang *et al.*, 2006) that have different fruit characters and peel colors. In the present study, the PPO from four cultivars of eggplant of *S. melongena* which had different fruit characters, fruit textures and peel colors were characterized and the effects of substrate specific, temperature, pH optimal, thermal stability on PPO activities were studied to identify the most appropriate eggplants as a source for natural browning agent.

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 every 10 seconds were recorded during 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 standard protein.

Evaluation of enzyme properties

a. 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 the substrates used 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.

b. Enzyme concentration

The enzymatic activities of each cultivar were studied by varying the extract concentrations from 1 to 20% (v/v), with reserved to the total volume of the reaction mixture. PPO activities were measured under the standard conditions as previously described. The measurements were carried out in triplicate.

c. Optimum pH

The enzyme activities were determined in the 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.

d. Optimum temperature

PPO activities of the enzyme extracts from different cultivars were studied between 2 and 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 the assays were performed in triplicate.

e. 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.

f. 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 slightly modified to maintain the final concentration of the substrate and the 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 of the substrate. The substrate solution was added prior to the 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 location 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) and the diphenolase or catecholase predominated the 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 in the previous study (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 condition as described in the standard assay condition. The four eggplant cultivars in this study can be divided into two groups according to their textures. 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.

Each cultivar PPO showed different specific activity (Table 1) when measured with 10 mM 4-methylcatechol as substrate. Under this condition, the values of K_m and V_{max} 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 the previous reports of the eggplant PPO (Perez-Gilabert and Garcia-Carmona, 2000; Dogan *et al.*, 2002; Concellon *et al.*, 2004).

Physiochemical properties of PPO

a. 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 higher activity for PPO of OW and OP.

PPO from eggplants also showed activity toward dopamine but not as high as the activity reached by the other substrates. It appears that substrate specific of eggplants 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 ΔOD min^{-1}), OG (K_m: 3.9 mM, V_{max}: 0.09 ΔOD min^{-1}), OW (K_m: 2.7 mM, V_{max}: 0.14 ΔOD min^{-1}) and OP (K_m: 2.9 mM, V_{max}:

0.22 ΔOD 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 set to a substrate for further standard assay.

b. Effect of enzyme concentration

The specific activities of eggplant PPO, using 4-methylcatechol as substrate showed a linear increase with the enzyme concentration (Fig. 2). PPO activity from each cultivar reached the steady period 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.

c. Effect of pH

Different pH optima were determined for PPO from each cultivar, using 4-methylcatechol as a substrate and are presented in Figure 3. These enzymes showed similar profile of PPO activities. 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 was rapidly decreased below pH 4.8 and slightly decreased from pH 6.0 (Concellon *et al.*, 2004).

d. 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 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 the oxidizing ability more than 50% when the temperature was over 60°C, similar to

those reported for another 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%.

e. 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, while it took longer period for inactivation of PPO from OG. Eggplants PPO was reported to be thermostable at low temperature, in the range of 0-20°C (Concellon *et al.*, 2004). 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 and 60°C, all the enzyme residuals still maintained more than 20% of their activities (Fig. 5). The results suggested that the eggplant PPOs from these cultivars were more heat tolerant than those of previous report (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 showed any reduction after being treated at 70°C for 5 min (Heimdal *et al.*, 1994).

f. 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), 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

compound, 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 were 28%, 12% and 33% inhibition, respectively. Adiculants are widely used in food processing to control browning as acids are naturally present in some edible food products such as ascorbic, citric, malic. Citric acid also functions as PPO inhibitor through its chelating action (Eskin *et al.*, 1971). Citric acid had 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 a 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 to use in fresh fruit and vegetable 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).

CONCLUSION

In the present study, fruit texture and peel color did not influence the enzymatic activity of eggplant PPO. Their 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 best conditions for these PPO to perform their browning reaction were at pH 6.0, 30°C with 1% (v/v) enzyme concentration and with 10 mM 4-methylcatechol as substrate. PPO from all cultivars showed thermal stable properties and had broad optimal temperature (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 eggplant PPOs found in

this study. Therefore, these compounds should be avoided if the maximum PPO enzymatic reaction is desired.

ACKNOWLEDGEMENTs

This research was financed by 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.

REFERENCES

Almeida, M. E., and J. N. Nogueira. 1995. The control of polyphenol oxidase activity in fruits and vegetables. A study of the interactions between the chemical compound used and heat treatment. Plant Foods for Human Nutrition, 47, 245-256.

Amorim, H. W. 1991. Significance of enzymes in non-alcoholic coffee beverage. p. 189-209 In P. F. Fox (ed), *Food enzymology*. New York: Elsevier Science Publishing.

Arslan, O., M. Erzengin, S. Sinan, and O. Ozensoy. 2004. Purification of mulberry (*Murus alba* L.) polyphenol oxidase by affinity chromatography and investigation of its kinetic and electrophoretic properties. Food Chemistry, 88, 479-484.

Beaulieu, M., G. M. Beliveau and M. Lacroix. 1999. Does rate effect of irradiation on the phenolic compounds, polyphenol oxidase and browning of mushrooms (*Agaricus bisporous*). Journal of Agricultural and Food Chemistry, 47(7), 2537-2543.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Cheriot, S. C., C. Billaud and J. Nicolas. 2006. Use of experimental design methodology to prepare Millard reaction products from glucose and cysteine-inhibitors of polyphenol oxidase from eggplant (*Solanum melogena*). Journal of Agricultural and Food Chemistry, 54, 5120-5126.

Concellon, A., M. C. Anon and A. R. Chaves. 2004. Characterization and changes in polyphenol oxidase from eggplant fruit (*Solanum melongena* L.) during storage at low temperature. Food Chemistry, 88, 17-24.

Dogan, M., O. Arslan and S. Dogan. 2002. Substrate specificity, heat inactivation and inhibition polyphenol oxidase from different aubergine cultivars. International Journal of Food Science and Technology, 37, 415-423.

Dogan, S., Y. Turan, H. Erturk and O. Arslan. 2005. Characterization and purification of polyphenol oxidase from artichoke (*Cynara scolymus* L.). Journal of Agricultural and Food Chemistry, 53, 776-785.

Eskin, N. A. M., H. M. Henderson and R.J. Townsend. 1971. Browning reaction in foods. p. 69-83. In N. A. M. Eskin, H. M. Henderson, and R. J. Townsend, Biochemistry of Food. New York: Academic Press.

Ferrar, P. H. and J. R. L. Walker. 1996. Inhibition of diphenol oxidases: A comparative study. Journal of Food Biochemistry, 20(6), 15-30.

Fujita, S. and T. Tono. 1988. Purification and some properties of polyphenol oxidase in eggplant (*Solanum melogena*). Journal of Science of the Food and Agriculture, 46, 115-123.

Heimdal, H., L. M. Larsen and L. Poll. 1994. Characterization of polyphenol oxidase from photosynthetic and vascular lettuce tissues (*Lactuca sativa*). Journal of Agricultural and Food Chemistry., 42, 1428-1433.

Jaworski, A. W. and C. Y. Lee. 1987. Fractionation of HPLC determination of grape phenolics. Journal of Agricultural and Food Chemistry., 35, 257-259.

Jiang, Y. and J. Fu. 1998. Inhibition of polyphenol oxidase and the browning control of litchi fruit by glutathione and citric acid. Food Chemistry, 62(1), 49-52.

Jiang, Y., G. Zauberman and Y. Fuchs. 1999. Purification and some properties of polyphenol oxidase of longan fruit. Food Chemistry, 66, 75-79.

Jiang, Y., L. Pen and J. Li. 2004. Use of citric acid for shelf life and quality maintenance of fresh-cut Chinese water chestnut. Journal of Food Engineering, 63(3), 325-328.

Lee, P. M., K. Lee and M. L. A. Karim. 1991. Biochemical studies of cocoa bean polyphenol oxidase. Journal of the Science of Food and Agriculture, 55(2), 251-260.

Martinez, M. V. and R. J. Whitaker. 1995. The biochemistry and control of enzymatic browning. Trends in Food Science and Technology, 6, 195-200.

Mayer, A. M. and E. Harel. 1979. Polyphenol oxidase in plants. Phytochemistry, 18, 193-215.

McEvily, A., R. Iyengar and S. Otwell. 1992. Inhibition of enzymatic browning in foods and beverages. CRC Critical Review of Food Science and Nutrition, 34, 253-273.

Panpeng, B. 2003. Collection and phylogenetics of some *Solanum* spp. in Thailand. Department of Horticulture, Faculty of Agriculture, M. Sc. (Agriculture) (p. 132). Chiang Mai: Chiang Mai University.

Perez-Gilabert, M. and Garcia-Carmona, F. 2000. Characterization of catecholase and cresolase activities of eggplant polyphenol oxidase. Journal of Agricultural and Food Chemistry, 48, 695-700.

Ullah, M. R. 1991. Tea. p 163-187. In P. F. Fox (ed). Food enzymology. New York: Elsevier Science Publishing.

Whitaker, B. D. and J. R. Stommel. 2003. Distribution of hydroxycinnamic acid conjugates in fruit of commercial eggplant (*Solanum melogena*) cultivars. Journal of Agricultural and Food Chemistry, 51, 3448-3454.

Yoruk, R. and Marshall, M. R. 2003. Phyiochemical properties and function of plant polyphenol oxidase: A review1. Journal of Food Biochemistry, 27(5), 361-422.

Zawitowski, J., C. G. Bilideris and N. A. M. Eskin. 1991. Polyphenol oxidase. p. 217-273. In D. S. Robinson, and N. A. M. Eskin, *Oxidative enzymes in foods*. New York: Elsevier.

Zhang, M. and D. Chen. 2006. Effects of low temperature soaking on color and texture of green eggplants. Journal of Food Engineering, 74(1), 54.

Table1. Eggplant cultivar characters, protein contents and specific PPO activity from different cultivars of eggplant *(Solanum melongena* L.) fruits.

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

[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.

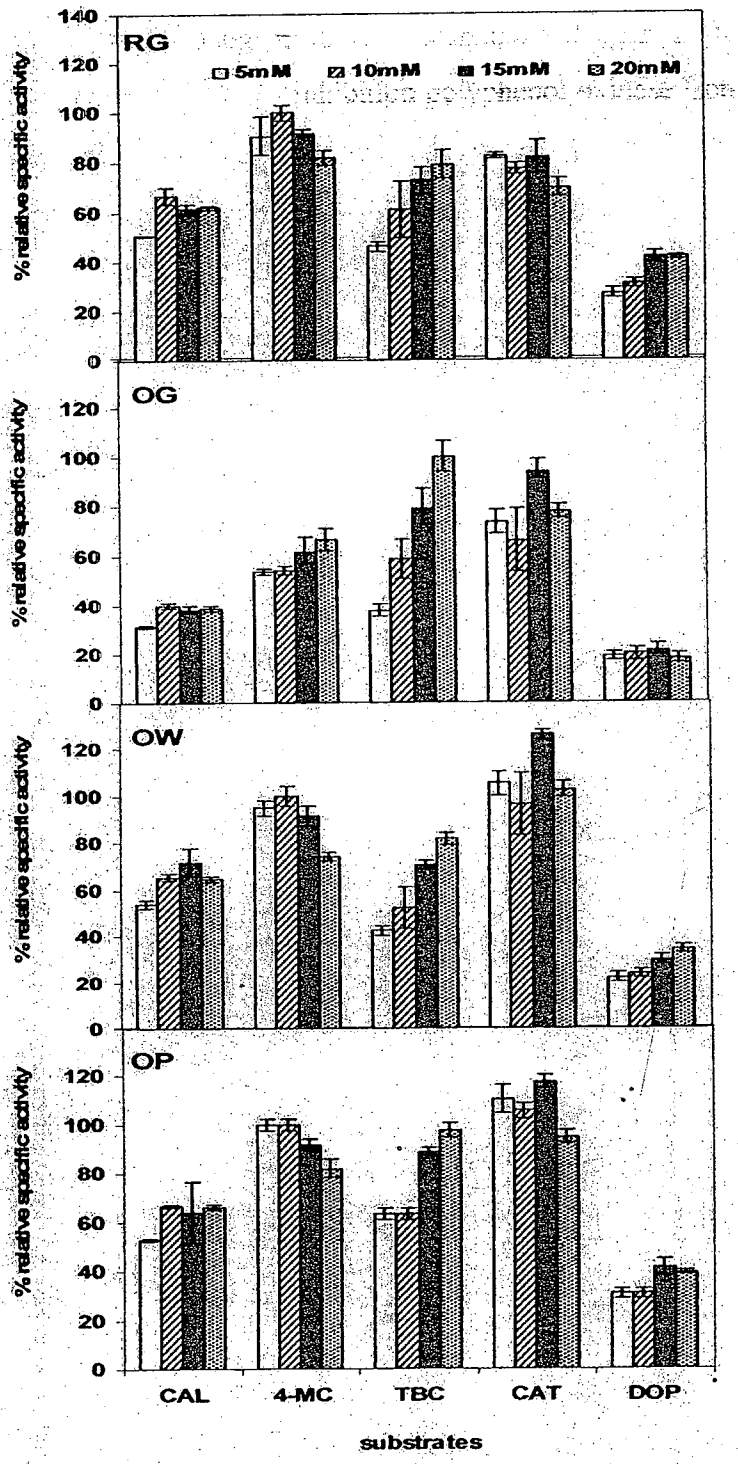


Figure 1. Substrate specificity (CAL: catechol, 4-MC: 4-methylcatechol, TBC: 4-tertbutylcatechol, CAT: catechin, DOP: dopamine) and concentration for PPOs from each cultivar of eggplant fruits. Values are means of triplicate determinations ± SE.

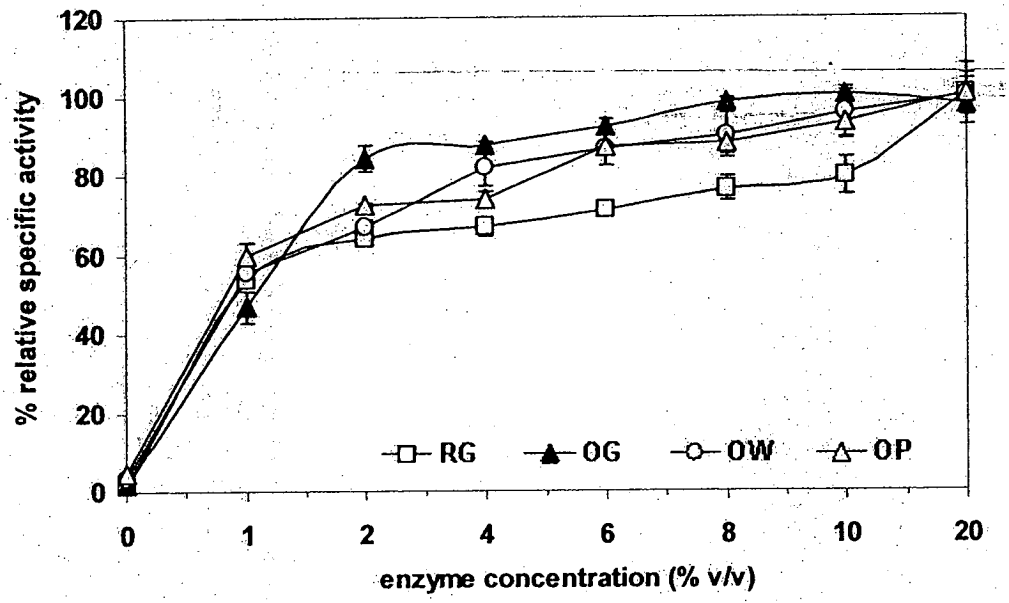


Figure 2. Effect of enzyme concentration on the specific activities of PPOs. Values are means of the relative activity expressed as a percentage of the maximum activity of each concentration ± SE.

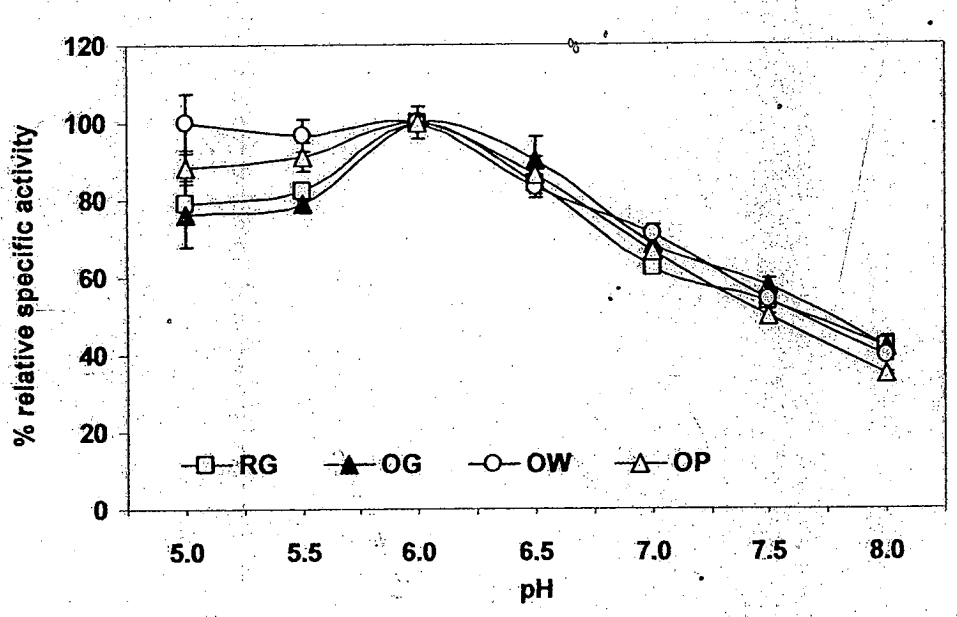


Figure 3. Effect of pH on the specific activities of PPOs. Values are means of triplicate determinations ± SE and represent as the relative activity expressed as a percentage from the highest PPO activity reached for each cultivar.

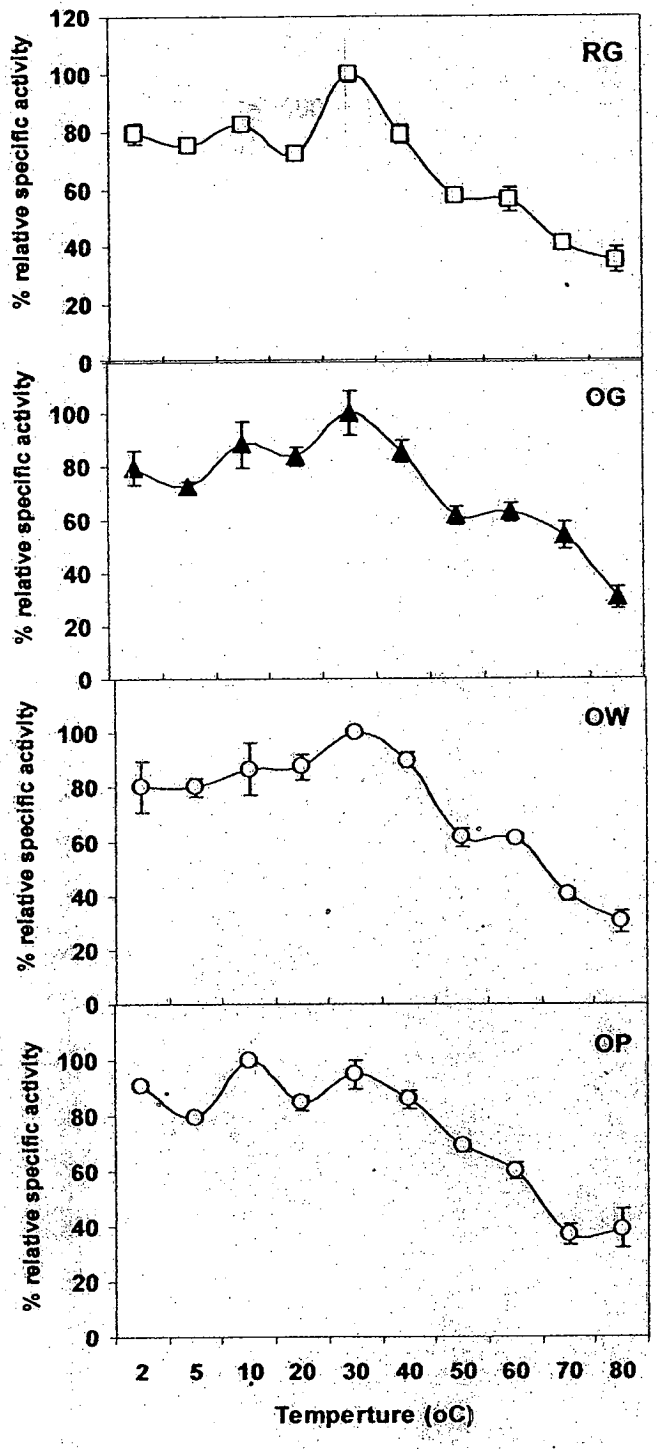


Figure 4. Optimal temperature for the specific activities of PPOs. Each data point represent the average of triplicate determinations ± SE and expressed as a percentage from the highest PPO activity reached for each cultivar.

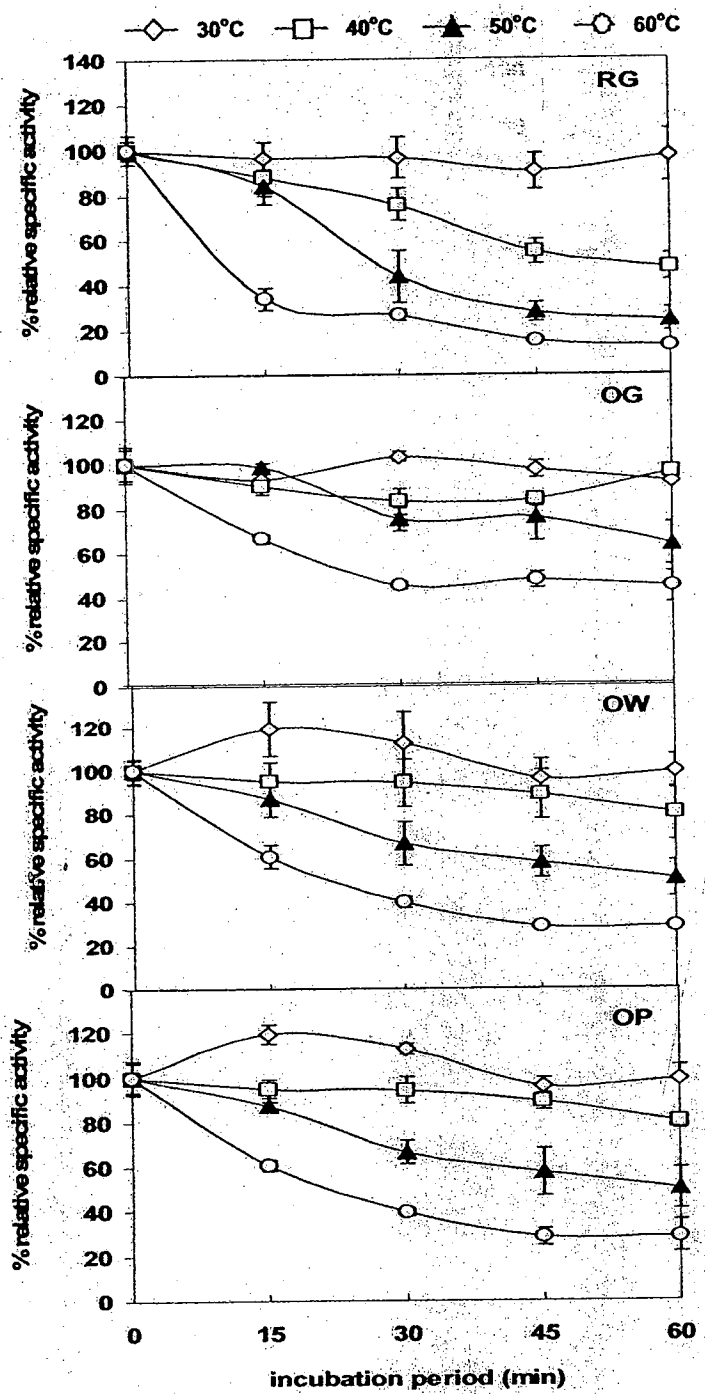


Figure 5. Thermal stability of PPOs with respect to incubation time at different temperatures. Values are means of triplicate determinations ± SE and expressed as a percentage of the initial activity.

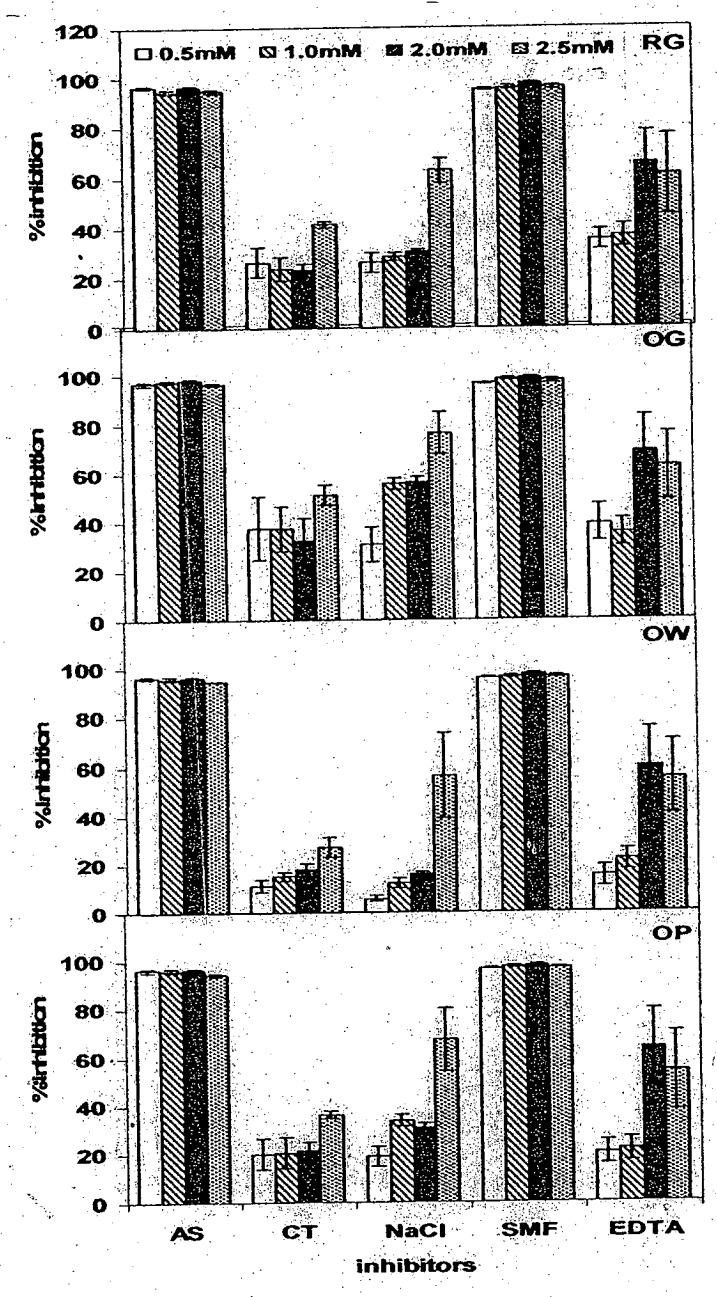


Figure 6 Effect of inhibitors on the residual enzymatic activity of PPO from different cultivars of eggplant fruits. Inhibitors were AS (ascorbic acid), CT (citric acid), NaCl (sodium chloride), SMF (sodium metabisulphite) and EDTA. Values are mean of triplicate determinations ± SE and represent as a percentage of inhibitory effect on the PPO activity.

1. วิธีการวัดปริมาณสารประกอบฟีนอล

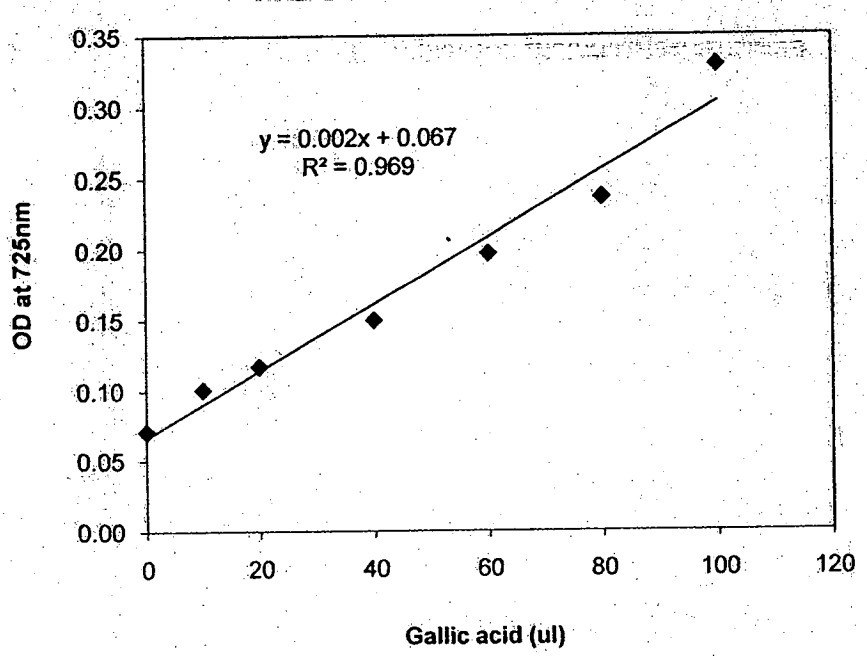
สร้างกราฟมาตรฐานสารละลายกรดแกลลิก โดยใช้กรดแกลลิก ความเข้มข้น 24 มิลลิกรัม ละลายด้วยเมทานอลความเข้มข้น 70% ปริมาณ 100 มิลลิลิตร เพื่อใช้เป็น stock solution นำสารละลายที่เตรียมไว้มาปรับความเข้มข้นให้อยู่ในช่วงความเข้มข้น 0 – 100 µl ต่อมิลลิตร เพื่อทำกราฟมาตรฐาน เตรียมสารละลายดังรายละเอียดในตาราง

ตารางที่ 1 การเตรียมสารละลายกรดแกลลิกมาตรฐานสำหรับใช้วัดด้วย microplate reader

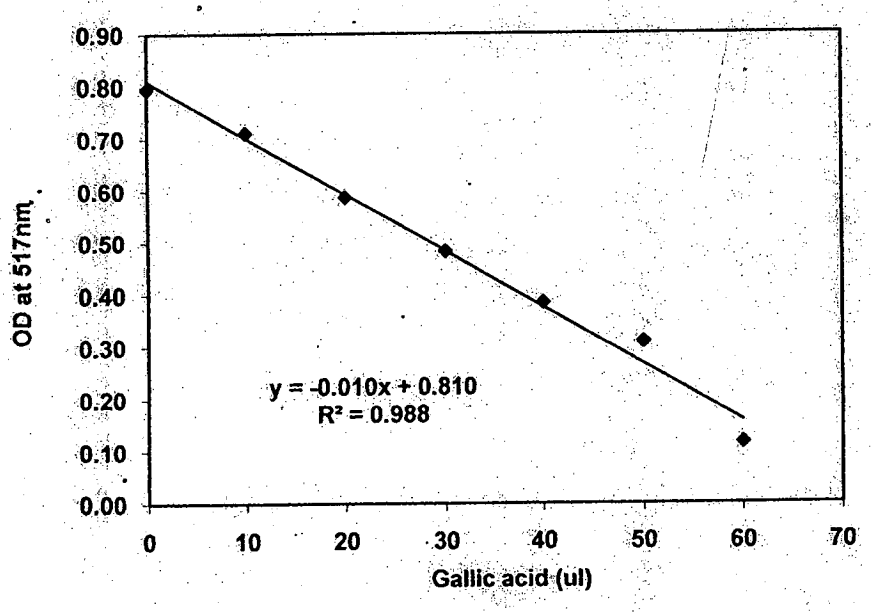
สาร	conc. 1	conc. 2	conc. 3	conc. 4	conc. 5	conc. 6	conc. 7
Gallic acid	0	1	2	3	5	6	8
DI H_2O	85	84	83	82	80	79	77
$NaCO_3$	29	29	29	29	29	29	29
Folin-Ciocalteau reagent	10	10	10	10	10	10	10
DI H_2O	77	77	77	77	77	77	77
ปริมาณ/well	200	200	200	200	200	200	200

นำสารละลายกรดแกลลิกที่เตรียมไว้มาเจือจางด้วยน้ำกำจัดไอออน 10 เท่า ใช้ความเข้มข้นละ 3 ซ้ำ เติมลงไปใน microplate ที่จะใช้ เติม $NaCO_3$ ความเข้มข้น 7.5% เขย่าให้เข้ากันบนเครื่องเขย่าสารแบบหมุน นาน 5 นาที หลังจากนั้นเติมสารละลาย Folin-Ciocalteau แล้วหุ้มด้วยกระดาษอลูมิเนียมฟอล์ยเพื่อป้องกันแสง นำไปวางบนเครื่องเขย่าสารนาน 2 ชั่วโมงที่อุณหภูมิห้อง นำสารละลายไปวัดค่าการดูดกลืนแสงที่ 725 nm ด้วยเครื่อง microplate spectrophotometer (Spectra MR, Dynex Technologies)

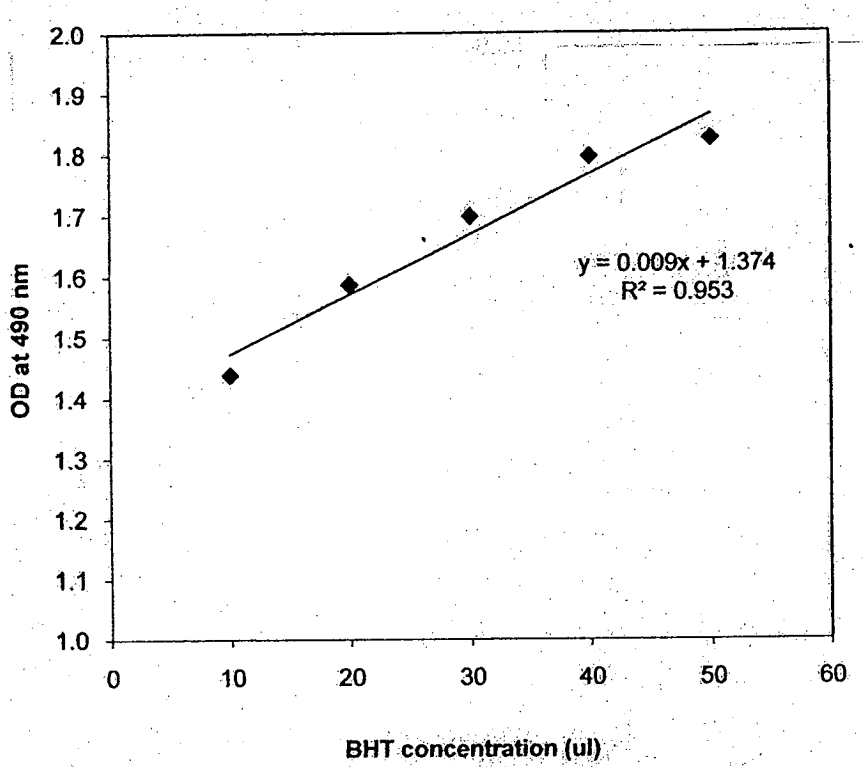
1. กราฟมาตรฐานของสารละลายกรดแกลิกสำหรับการวัดปริมาณสารประกอบฟีนอลทั้งหมด



2. กราฟมาตฐานกรดแกลลิกสำหรับการวัดกิจกรรมต้านปฏิกิริยาออกซิเดชั่นด้วยวิธี DPPH assay



3. กราฟมาตรฐานสำหรับβ-carotene bleaching assay เมื่อใช้ BHT เป็นสารมาตรฐาน



4. กราฟมาตรฐานสำหรับ ABTS$^{\bullet+}$ assay เมื่อใช้ Trolox เป็นสารมาตรฐาน