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Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (*Capsicum annuum* L.) pericarb

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Abstract: The activities of polyphenol oxidase (PPO) and peroxidase (POD) in hot pepper (*Capsicum annuum* L.) pericarp were evaluated using spectrophotometric method. The enzymes were extracted from the pepper pericarp 5 g with 20 mL of 0.1 M phosphate buffer solution pH 7. PPO and POD activities were determined using catechol and guaiacol as a substrate, respectively. The effects of the amounts of enzyme extract, substrate concentration, hydrogen peroxide concentration (only for POD), pH and temperature were investigated. The PPO activity was linearly increased with an increasing the amount of the enzyme extract between 20 and 200 µL. While the POD activity was also found in the same manner between 25 and 100 µL. The highest activity of PPO was obtained when using catechol concentration of 33 mM. That of POD was maximal optimized with 18 mM guaiacol and 4.9 mM H_2O_2. The optimum temperatures were 30°C for PPO and 40°C for POD. These optimum conditions were used to determine both enzyme activities in some varieties of hot pepper samples.

Key words: Polyphenol oxidase, peroxidase, enzyme activity, *Capsicum annuum* L.

Introduction

Polyphenol oxidase (PPO) is a copper-containing enzyme which is probably present in all plants. It is widely distributed enzyme involved in the biosynthesis of melanins in animals and in the browning of plants. The enzyme catalyzes the oxidation of phenolic compounds to form corresponding quinone intermediates which polymerize to form undesirable pigment. It catalyzes two types of the oxidative reaction involving molecular oxygen: the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones, which lead to the formation of black or brown pigments (Lee *et al*., 2007; Gawlik-Dziki *et al*., 2007).

Peroxidase (POD) is an oxidoreductase that is directly involved in many plant functions such as hormone regulation, defence mechanisms, indolacetic degradation and lignin biosynthesis (Serrano-Martínez *et al*., 2008). It catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atom. POD involved in enzymatic browning since diphenols may function as reducing substrate in this reaction (Chisari *et al*., 2007; Serrano-Martínez *et al*., 2008). The involvement of POD in browning is limited by the availability of electron acceptor compounds such as superoxide radicals, hydrogen peroxide and lipid peroxidase.

Enzymatic browning is a significant problem in a number of fruits and vegetables such as strawberry (Chisari *et al*., 2007), grape (Muñoz *et al.*, 2004), potato (Lee and Park, 2007), and lettuce (Gawlik-Dziki *et al*., 2007). The discoloration in fruits and vegetables by enzymatic browning, resulting from conversion of phenolic compounds to *o*-quinones which subsequently polymerize to be a brown or dark pigment. The enzymes involved these processes are PPO and POD (Jiang *et al.*, 2004). Because PPO and POD are the main enzymes involved the phenolic oxidation of many fruits and vegetables, their activities have attracted much attention. The relationship between the degree of browning and PPO activity were studied in processing apple varieties to provide reference for raw material selection (Ye *et al*., 2007). The kinetic characteristic and thermal inactivation

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property of POD extracted from red sweet pepper (*Capsicum annuum* L.) was studied. The enzyme was partially purified and its activity was evaluated using ABTS as substrate (Serrano-Martínez *et al.*, 2008). Since residual enzyme activities were assumed to cause capsaicinoid losses, POD activity in hot chili peppers (*Capsicum frutescens* L.) were investigated a long with the changes in capsaicinoid contents (Schweiggert *et al.*, 2006).

Peppers are popular vegetables because of the combination of color, taste and nutritional value. Fresh peppers are good source of vitamin C and E, provitamin A and carotenoids (Chatterjee *et al*., 2007; Deepa *et al.*, 2007). Moreover, the red pepper fruit (*Capsaicum annuum* L.) has been used for many years as a source of pigments to add or change the color of foodstuffs, making them more attractive and acceptable for the consumer. Thus, the aim of the present study was to evaluate PPO and POD activities in hot pepper pericarp. The optimum conditions for determination of both enzyme activities by spectrophotometric method were investigated including the amounts of enzyme extract, concentration of substrate, pH and incubation temperature.

Materials and Methods

Chemicals

Polyvinylpyrrolidone was obtained from Fluka, UK. Catechol and guaiacol were purchased from Across, USA. Di-sodium hydrogen phosphate was purchased from Fisher chemical, UK. Potassium dihydrogen phosphate was purchased from Scharlau Chemie S.A., Spain. Tri-sodium citrate was obtained from Ajax chemical, Australia. Citric acid and phosphoric acid were AR grade, Carlo Erba, Italy.

Materials

Pepper fruits used in this study were obtained from a local market in Khon Kaen, Thailand. The pepper fruits were washed several times with tap water and the seeds and peduncle were removed. The pericarp was homogenized by using homogenizer (Moulinex Optiblend 2000, France) for 2 min and stored at 4°C for further experiments. The codes and descriptions of the pepper samples used in this study are summarized in Table 1. The photographs of some samples are shown in Figure 1.

Enzyme extraction

All steps of enzyme extraction were carried out at 4°C. Five grams of the homogenized pepper pericarp were extracted with 0.1 M phosphate buffer pH 7 containing 5 g of polyvinylpyrrolidone using magnetic stirrer for 15 min. The homogenate was filtered through Whatman No.41 filter paper and then centrifuged at 2,500 rpm (1000 series centifugal, England) for 20 min. The supernatant was filtered through Whatman No.42 filter paper and collected as an enzyme extract.

Table 1. Lists of some hot pepper samples used

Code	Sample type	Part used
RP1	Red pepper fruits	Fleshes
RP2	Red pepper fruits	Fleshes
RP3	Red pepper fruits	Fleshes
RP4	Red pepper fruits	Fleshes
RP5	Red pepper fruits	Fleshes
RF5	Red pepper fruits	Fruits
OP1	Orange pepper fruits	Fleshes
GP1	Green pepper fruits	Fleshes
GP2	Green pepper fruits	Fleshes
GP3	Green pepper fruits	Fleshes
GP4	Green pepper fruits	Fleshes
GP5	Green pepper fruits	Fleshes
GP6	Green pepper fruits	Fleshes
GP7	Green pepper fruits	Fleshes
GF7	Green pepper fruits	Fruits
GF8	Green pepper fruits	Fruits
FC	Fish sauce with red chili	Pepper pieces
VC	Vinegar with green chili	Pepper pieces

Enzyme assays

PPO activity was determined using a spectrophotometric method based on an initial rate of increase in absorbance at 410 nm (Soliva *et al.*, 2001). Phosphate buffer solution pH 7 (0.1 M, 1.95 mL), 1 mL of 0.1 M catechol as a substrate and 50 μL of the enzyme extract were pipetted into a test tube and mixed thoroughly. Then the mixture was rapidly transferred to a 1-cm path length cuvette. The absorbance at 410 nm was recorded continuously at 25°C for 5 min using ultraviolet-visible spectrophotometer, Agilent, Germany.

POD activity was assayed spectrophotometrically at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide (Díaz *et al.*, 2001). The reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H_2O_2, 2.66 mL of 0.1 M phosphate buffer pH 7 and 40 μL of the enzyme extract. The blank sample contained the same mixture solution without the enzyme extract.

RP2

RP3

OP1

GP2

GP3

GP6

Figure 1. The photographs of some hot pepper samples used

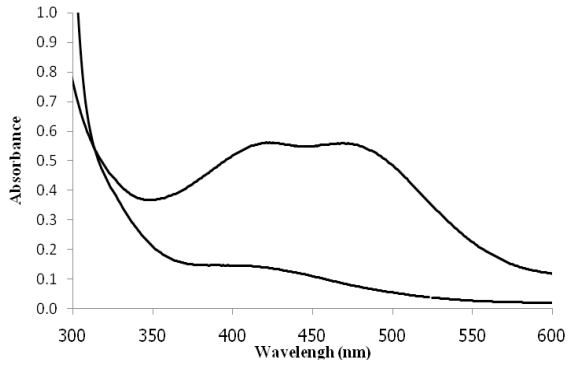

Figure 2. Absorption spectra, at 25°C, obtained for (a) the oxidation of catechol by hot pepper PPO and (b) the oxidation of guaiacol by hot pepper POD. The reaction mixture of both enzymes are described in the text.

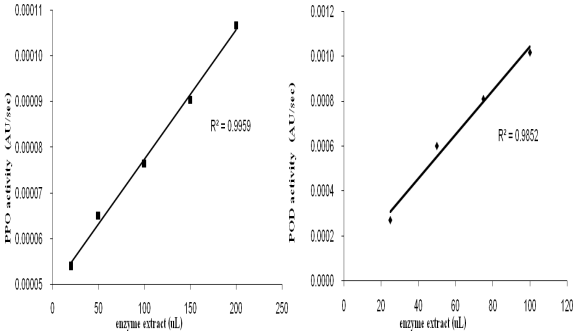

Figure 3. Effect of amounts of the enzyme extract on both PPO (a) and POD (b) activities.

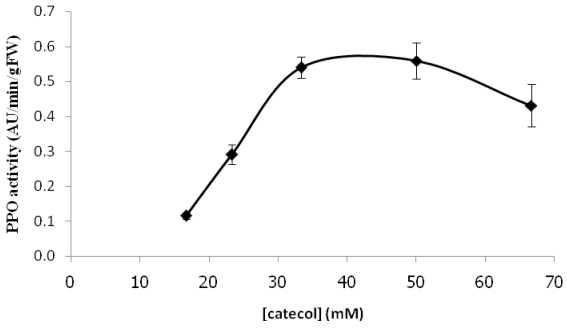

Figure 4. Effect of catechol concentration on the hot pepper PPO activity.

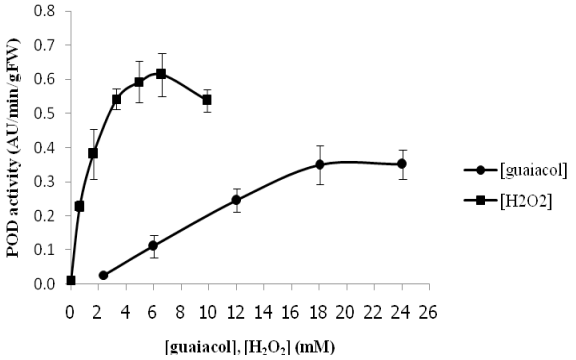

Figure 5. Effect of guaiacol and hydrogen peroxide concentrations on the hot pepper POD activity.

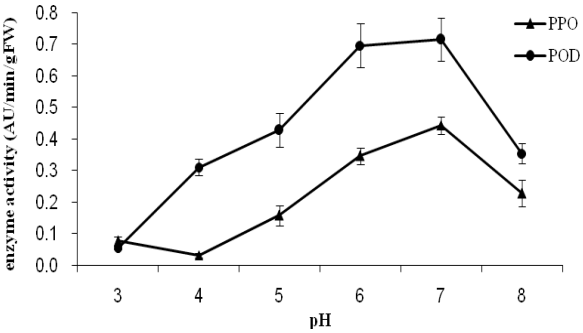

Figure 6. Effect of pH on the hot pepper PPO and POD activities.

Effect of amounts of enzyme extract on enzyme activity

The activity of PPO and POD as a function of amounts of enzyme extract was investigated. PPO activity was assayed at various amounts of the enzyme extract from 20 to 200 μL by mixing with 2 mL of 0.1 M catechol, and 1 mL of 0.1 M phosphate buffer pH 7. POD activity was also performed using the amounts of the enzyme extract from 25 to 100 μL. The solution of the reaction mixture contained 0.1 mL of 1% (v/v) H_2O_2, 0.1 mL of 4% (v/v) guaiacol, and 2.78 mL of 0.1 M phosphate buffer pH 7.

Effect of substrate concentration on enzyme activity

The enzyme activities were measured in a quartz cuvette of 3 mL volume. PPO activity was proceeded by mixing 50 μL of the enzyme extract, 0.1 M catechol and 0.1 M phosphate buffer pH 7 at a selected volume. POD activity was observed by using the mixture containing 40 μL of the enzyme extract, 0.1 mL of 1%(v/v) H_2O_2, 4%(v/v) guaiacol and 0.1 M phosphate buffer pH 7 at a selected volume. The

effect of H_2O_2 concentration on POD activity was studied using the reaction system consisted of 40 µL of the enzyme extract, 0.15 mL of 4%(v/v) guaiacol, 1%(v/v) H_2O_2 and 0.1M phosphate pH 7 at a selected volume. In each measurement, the final volume of the reaction solution in a quartz cuvette was 3 mL.

Effect of pH on enzyme activity

The activity of PPO and POD were determined at pH values of 3, 4, 5, 6, 7 and 8 using 0.1 M citrate buffer (pH 3-5) and phosphate buffer (pH 6-8). The optimum pH for PPO and POD were obtained using catechol and guaiacol as substrate, respectively. The effect of pH on PPO activity was observed by using the reaction mixture containing 1 mL of 0.1 M catechol, 1.95 mL of 0.1 M buffer solution and 50 µL of the enzyme extract. For POD, the reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H_2O_2, 2.66 mL of 0.1 M buffer solution and 40 µL of the enzyme extract.

Effect of temperature on enzyme activity

PPO and POD activities were determined at 20, 30, 40, 50, 60 and 70°C. The substrate and buffer solutions were incubated for 5 min at various temperatures from 20 to 70°C before adding of the enzyme extract. Spectrophotometric measurement for 5 min was carried out at 25°C. The activity of PPO under optimum temperature was determined by adding 1 mL of 0.1 M catechol, 1.95 mL of 0.1 M phosphate buffer pH 7 and 50 µL of the enzyme extract. For POD, the reaction mixture contained 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H_2O_2, 2.66 mL of 0.1 M phosphate buffer pH 7 and 40 µL of the enzyme extract.

Results and Discussion

Optimization conditions for enzyme activity measurements

PPO and POD are oxidative enzymes which catalyze the oxidation of phenolic substrates mainly due to enzymatic browning (Jiang *et al.*, 2004). They catalyze the oxidation of phenolic compounds to *o*-quinone which polymerize to form undesirable pigments (Chisari *et al.*, 2007). In this study, catechol and guaiacol were used as the substrate for PPO and POD, respectively. The enzymatic oxidation of catechol by PPO/O_2 transformed the substrate into yellow products with a maximum absorbance at 410 nm. The enzymatic oxidation of guaiacol by POD/H_2O_2 changed the substrate into orange-pink products with a maximum absorbance at 470 nm (Figure 2).

The effect of various amounts of the enzyme extract on PPO and POD activities was studied and the result was shown as the rate of substrate oxidation by the enzymes. The substrate oxidation was found to be dependent on the amounts of the enzyme extract. An increase in enzyme concentrations resulted in the corresponding linear increase in both enzyme activities (Figure 3), in the enzyme concentration range assayed (20 to 200 µL for PPO and 25 to 100 µL for POD).

As shown in Figure 2, the oxidation of catechol by hot pepper PPO generated products which had absorbance maximum at 410 nm. Thus, the optimal catechol concentration was determined by measuring the increase in absorbance at 410 nm, using different amounts of the substrate (Figure 4). As expected, an increase in the substrate concentration resulted in an increase in pigment formation. The rate of which stayed practically constant at saturating catechol concentration. Therefore, the concentration of 33.3 mM catechol was routinely chosen because at higher concentrations of the substrate did not significantly affect the formation of the *o*-quinone intermediate.

When the oxidation of guaiacol by hot pepper POD was carried out in the presence of H_2O_2, the quinone intermediate formed gave absorbance maximum at 470 nm. The optimal guaiacol concentration was determined and the results are shown in Figure 4. The optimal concentration of guaiacol was found to be 18 mM. In addition, when the H_2O_2 concentration was increased, at a fixed saturating concentration of guaiacol, POD exhibited the highest activity at 4.9 mM of H_2O_2 (Figure 5).

The activity of PPO and POD were measured at different pH values using catechol and guaiacol as substrate, respectively. As shown in Figure 6, the optimum pH 7 of both enzymes was obtained. It is known that the optimum pH for any enzymes depends on plant materials and substrate in the activity assay. In general, most plants show maximum enzyme activity at or near neutral pH. Different optimum pH values for both enzymes obtained from various sources and substrates used have been reported. The optimum pH values are 6.8 and 5.5 for butter lettuce PPO using 4-methycatechol and catechol as substrates, respectively (Gawlik-Dziki *et al*., 2007), pH 6.5 for longan fruit PPO using 4-methycatechol as substrate (Jiang, 1999), pH 6.0-8.5 for kiwifruit POD using *p*-phenyllenediamine as substrate (Fang *et al*., 2008) and pH 6.0 for spring cabbage POD using guaiacol as substrate (Belcarz *et al.*, 2008).

The optimum temperature for enzyme activity usually depends on experimental conditions. Generally, the reaction rate decreases because of thermal denaturation when the temperature is increased. This situation is similar for most enzymes.

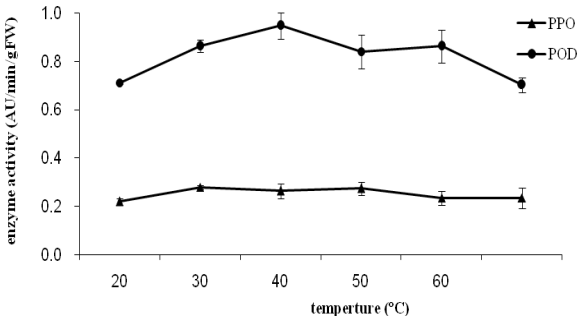

Figure 7. Effect of temperature on the hot pepper PPO and POD activities.

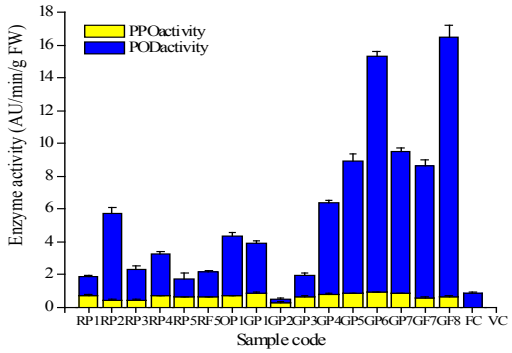

Figure 8. PPO and POD activities in some hot pepper samples.

Table 2. Enzyme activities in hot pepper samples based on fresh weight.

Code	PPO activity[a] (ΔA/min/gFW)	POD activity[a] (ΔA/min/gFW)
RP1	0.730 ± 0.030	1.13 ± 0.063
RP2	0.402 ± 0.078	5.29 ± 0.363
RP3	0.429 ± 0.088	1.89 ± 0.181
RP4	0.696 ± 0.040	2.54 ± 0.189
RP5	0.612 ± 0.021	1.11 ± 0.348
RF5	0.622 ± 0.037	1.54 ± 0.058
OP1	0.728 ± 0.015	3.60 ± 0.201
GP1	0.846 ± 0.094	3.06 ± 0.149
GP2	0.292 ± 0.085	0.229 ± 0.028
GP3	0.609 ± 0.068	1.33 ± 0.148
GP4	0.787 ± 0.087	5.57 ± 0.137
GP5	0.848 ± 0.022	8.08 ± 0.451
GP6	0.929 ± 0.007	14.4 ± 0.224
GP7	0.839 ± 0.015	8.69 ± 0.171
GF8	0.617 ± 0.057	15.8 ± 0.760
FC	n.d.	0.899 ± 0.063

[a]activity is expressed as mean ± SD (n = 3)
n.d. : not detectable

Temperature dependence in the enzyme activities is presented in Figure 7. It was found that the highest activity of PPO and POD were obtained at 30°C and 40°C, respectively. PPO showed the highest activity at 30°C, and its activity decreased slightly between 40 and 70°C. The POD activity increased when the temperature was increased from 20 to 40°C, and then decreased probably due to denaturation of the enzyme at higher temperatures. From previous studied, the temperature at which PPO showed the highest activity was in the range of 25-30°C, and then decreased at temperature above 40°C (Doğan and Doğan, 2004). In case of POD, the enzyme was highly active up to 40°C and lost its activity at higher temperatures (Fatima, 2007; Saraiva, 2007; Belcarz, 2008). From the obtained results, the optimum temperature of the both enzymes was found between 30°C and 40°C. Thus, we determined the enzyme activities of pepper samples at ambient temperature (30 ± 3 °C).

Determination of enzyme activity in hot pepper samples

The proposed method was applied for the determination of PPO and POD activities in hot pepper pericarp. There are various kinds of green, orange and red pepper fruits and two kinds of commercially available samples, sliced red chilli in fish sauce and sliced green chilli in vinegar. The results are shown in Table 2. The PPO activities in the pepper samples were found to be 0.29-0.93 AUmin^{-1}g^{-1} fresh weight, whereas the POD activities were ranged of 0.23-16 AUmin^{-1}g^{-1} fresh weight. The highest activities of PPO and POD were found in the pepper sample GP6 and GF8, respectively. The lowest activity of both enzymes was found in the sample GP2. Only the POD activity of the sliced red chilli in fish sauce was detectable. But both enzyme activities in the sliced green chilli in vinegar were not detectable. According to the results, both enzyme activities in almost immaturity stage of green pepper samples were found to be higher than those in the maturity stage of the red ones. It can be suggested that the activity of these enzymes was depended on the stage of maturity. In addition, the enzyme activities of the pepper fruits can be varied with genotype, conditions during growth, post harvest handing and shelf life

(Boonsiri *et al.*, 2007).

Figure 7 shows the relationship between PPO and POD activities in hot pepper samples. It is shown that both enzyme activties in almost fruit samples have a similar tendency. The samples with high PPO activity also have high POD activity, except the sample GP2. Generally, the oxidation of phenolics in plant materials is mainly catalyzed by PPO and POD at which may also contribute in this reaction. It has been reported that the oxidation of phenolic compounds by PPO could generate H_2O_2 and the POD kept the generated H_2O_2 to get further oxidation of the phenolic compounds. Thus, both enzyme activities would function the same trends in the plant materials.

Conclusion

A spectrophotometric method for the measurement of enzymatic activity of hot pepper PPO and POD was developed. The results are calculated based on fresh weight ranging from 0.29-0.93 and 0.23-16 $AUmin^{-1}g^{-1}$, for PPO and POD, respectively, found in these pepper samples. The relationship between PPO and POD activities in the samples were investigated resulting in similar tendency for both enzymes in almost fruit samples.

Acknowledgements

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