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Some Characteristics of Polyphenol Oxidase and Peroxidase from Taro *(Colocasia antiquorum)*

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Abstract: The heat inactivation kinetics of taro polyphenol oxidase (PPO) and peroxidase (POD) in the temperature range of 50°–80°C followed the first–order kinetic model. Both enzymes possessed two isoenzymes with varying heat stabilities. In the range of 60°–70°C, heat stable isoenzymes accounted for 33–34% of POD and 67–72% of PPO. E_a and z values of heat stable isoenzymes were, respectively, 19.4 kcal.mol^{-1} and 25.9°C for POD, and 21 kcal.mol^{-1} and 25.5°C for PPO. The pH optimum was 5.9 for POD and 6.5 for PPO. POD was densely located on the surface of taro tubers whereas PPO was located more to the center. Taro PPO possessed both catechol oxidase and phloroglucinol oxidase activities but no laccase activity. Inhibition of PPO by EDTA, SO_2, NaCl and ascorbic acid was also determined.

Taro *(Colocasia antiquorum)* Polifenol Oksidaz ve Peroksidaz Enzimlerinin Bazı Niteliklerinin Belirlenmesi

Özet: Taro yumrularından ekstrakte edilmiş peroksidaz (POD) ve polifenol oksidaz (PPO) enzimlerinin 50°–80°C arasındaki ısıl inaktivasyonunun birinci dereceden bir reaksiyon kinetiğine uygun olarak geliştiği saptanmıştır. Her iki enzimin, ısıl dirençleri birbirinden farklı iki izoenzimden oluştuğu belirlenmiştir. 60°–70°C sıcaklık aralığında, ısıl direnci yüksek olan fraksiyonun oranı POD için; %33–34 ve PPO için %67–72 olarak bulunmuştur. Diğer taraftan bu enzimlerin E_a ve z değerlerinin sırasıyla; POD için 19.4 kcal.mol^{-1}, 25.9°C ve PPO için ise 21 kcal.mol^{-1}, 25.5°C düzeyinde olduğu saptanmıştır. Aynı şekilde, pH optimumu POD için 5.9 ve PPO için 6.5 olarak belirlenmiştir. Bu enzimlerin yumrudaki dağılımı da incelenmiş ve POD enziminin yumruların yüzey kısımlarında, buna karşın PPO enziminin ise daha çok merkez kısımlarında yoğunlaştığı saptanmıştır. PPO enziminin kateşol oksidaz ve floroglusinol oksidaz aktivitesi içermesine karşı lakkaz aktivitesine sahip olmadığı görülmüştür. EDTA, SO_2, NaCl ve askorbik asidin PPO enzimi üzerindeki inhibe edici özellikleri de belirlenmiştir.

Introduction

Taro *(Colocasia antiquorum)* is known in the Pacific islands as "taro", in the West Indies as "eddo" or "dasheen" and in West Africa as "old cocoyam." It is a root crop grown in many parts of the wet tropics as well as in some parts of the Mediterranean. The edible part is the corm formed underground by a thickening of the base of stem. Some varieties also produce subsidiary tubers called "cormels." The starch of corms is very finely grained and easily digestible (1).

Corms are highly susceptible to enzymic browning. Mechanical injury during storage and processing results in the brown discolaration of corms. This indicates a highly active polyphenol oxidase enzyme and/or a highly reactive polyphenol content of the corm tissue. It is generally accepted that two kinds of PPO enzyme exist in nature. One of these enzymes is called "catechol oxidase" (E.C.1.10.3.1) which oxidizes o–diphenols to quinones (catecholase activity) and also hydroxylates monophenols to o–diphenols (cresolase activity). The other PPO is "laccase" (E.C.1.10.3.2) capable of oxidizing both o–and p–diphenols. Other than these PPO enzymes, Fujita et al. (2) found that there is a third PPO enzyme oxidizing only phloroglucinol (1, 3, 5–trihydroxybenzene) and they called it "pholoroglucinoL oxidase" (PhO). The oxidation of phenolic compounds present in the plant tissues by PPO enzymes leads to the formation of underised brown pigments and off–flavored products (3, 4).

PPO is generally used as an indicator enzyme for the adequacy of heat treatment of fruit purees (5, 6). However, it has rarely been used as a blanching indicator for vegetables. On the other hand, peroxidase (POD; EC.1.11.1.7), another oxidoreductase enzyme, is widely used for this purpose because it is considered to be the

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most heat stable enzyme in the vegetable tissues and is present in almost all vegetables (7, 8, 9). Compared with PPO, POD causes browning to a lesser extent because it needs the presence of hydrogen peroxide or an organic peroxide to oxidize phenolics (6, 8, 10). On the other hand, there is an empirical relationship between the prevention of off–flavor development in frozen vegetables and inactivation of POD (6, 7, 8, 11). Therefore, the characteristics of these enzymes have a technological importance for the processing of vegetables. In this research, the heat inactivation kinetics, optimum pH and certain other characteristics of taro POD and PPO, including the location of these enzymes in corms, were investigated.

Materials and Methods

Material

Corms were purchased from the local market in Lefkoşa (T.R.N.C.) in February, 1996 and stored at–30°C until used.

Methods

Extraction of enzymes

Acetone powder was first prepared from the corms according to the procedure outlined by Coseteng and Lee (12). For enzyme extraction, 3 g of acetone powder was suspended into 50 mL of cold 0.05 M Na–phosphate buffer, pH 6.8. The suspension was then filtered through five layers of cheesecloth and centrifuged at 5000 g for 25 min. The supernatant was used as the crude enzyme extract for this study after diluting 1:8 (v/v) with 0.05 M Na–phosphate buffer, pH 6.8.

Assay for PPO activity

PPO enzyme acitvity was measured spectrophotometrically by monitoring the increase in absorbance at 420 nm at 15 sec time intervals with a PYE UNICAM SPG–550 spectrophotometer. The reaction mixture contained 0.5 mL of 0.5 M catechol and 2 mL of enzyme extract. All assays were performed at 30°C in duplicate. The reaction rate was calculated from the slope of the linear portion of an absorbance vs time curve.

Assay for POD activity

POD activity was measured spectrophotometrically after a minor modification of the method described by Heil et al. (13). The reaction mixture contained 0.2 mL of 0.5% (w/v) o–tolidine, 0.2 mL of 0.1% H_2O_2 and 2 mL of enzyme extract. All assays were performed at 30°C in duplicate. To determine POD activity, the increase in absorbance was recorded at 660 nm at 5 sec time intervals (14). The reaction rate was calculated from the slope of the linear portion of an absorbance vs time curve.

Heat inactivation studies

Heat inactivation studies were carried out in the temperature range of 50°–80°C by using Thermal Death Time (TDT) tubes. To reduce lag time, TDT tubes with 4 mL of 0.05 M Na–phosphate buffer at pH 6.8 were heated to inactivation temperature. 0.5 mL of enzyme extract was pipetted into TDT tubes, mixed with a vortex and immersed in a water bath (Mess–Technic Ultra Thermostat, ±0.2 °C). After holding for various lengths of time, the tubes were immediately placed into an ice bath. POD and PPO activities were measured by the spectrophotometric methods described earlier. The percentage of activity remaining after each treatment was calculated from the initial activity.

Effect of pH

To determine the pH optimum for POD and PPO, activity measurements were carried out in the pH range of 5.5–7.5 using 0.05 M Na–phosphate buffer.

Location of enzymes

Test papers were used to determine the enzyme location in the corms. Test paper for POD was prepared and used according to the method described by Morris (15). Test paper for PPO was, on the other hand, prepared by dipping Whatman # 1 filter paper into 0.5 M catechol solution. Excess liquid was removed from the test papers onto filter paper. Test papers for PPO were prepared prior to each assay and used wet. To determine enzyme location, taro tubers were sliced horizontally and pressed onto test papers. On the test papers, the density of blue color development for POD and brown color for PPO were proportional to the enzyme activity present.

Substrate specificity of PPO

To determine the substrate specificity of PPO, 0.1 M of phenol, p–cresol, hydroquinone, catechol and phloroglucinol was prepared. The reaction mixture for activity measurements contained 0.2 mL of 0.1 M various substrates, 1 mL of 0.1 M Na–phosphate buffer at pH 6.8 and 1 mL of enzyme extract. The reaction rates were measured at the wavelengths given in Table 2. Enzyme activity was determined as $\Delta_{OD}.min^{-1}.mL^{-1}$ and compared with the rate of catechol oxidation.

Inhibition of PPO

EDTA, SO_2, NaCl and ascorbic acid at varying concentrations were prepared in 0.5 M Na–phosphate buffer at pH 6.8. 4 mL of inhibitor solution was then

mixed with a 0.5 mL of enzyme extract and incubated for 5 min at 30°C. For activity measurement, 0.5 mL of 0.5 M catechol was added into 2 mL of inhibitor–enzyme mixture. Enzyme activity was measured according to the method given above.

Results and Discussion

Heat inactivation

Heat inactivation curves of taro PPO and POD are shown in Figures 1 and 2, respectively. Inactivation of both PPO and POD followed the first–order kinetic model. Each inactivation curve consisted of an initial straight line with a steep slope (heat labile part) and a final straight line with a shallow slope (heat stable part). This kind of inactivation pattern indicates the presence of two different isoenzymes varying in heat stabilities. The percentage of heat stable isoenzyme was estimated by extrapolating the heat stable portion of the inactivation curve to zero time (16, 17, 18). The percentage of heat stable isoenzyme of POD and PPO at various temperatures is shown in Table 1. It is clear that more than half of the PPO enzyme consisted of the heat stable isoenzyme. However, the amount of heat stable isoenzyme for POD was less than that of PPO. As shown in Figures 1 and 2, the heat labile parts of both curves showed rapid inactivation. Therefore, all heat inactivation parameters in this study were calculated for heat stable isoenzymes. Arrhenius and TDT methods (19) were used to determine the heat stability of taro POD and PPO enzymes.

Calculated z values for taro POD and PPO were 25.9°C (r=0.974) and 25.5°C (r=0.999), respectively. The z value of 25.9°C for POD inactivation was in agreement with the z value of POD from other sources. The z value of 20.5°C for POD/PPO complex was found for apricots by Heil et al. (13). Naveh et al. (18) reported a z value of 33°C for the heat stable isoenzyme of corn POD. Comparing with values in the literature (6, 20), a z value of 25.5°C for taro PPO is considered relatively high.

Respective E_a values for heat inactivation of taro PPO and POD were 21 kcal.mol^{-1} (r=0.999) and 19.4 kcal.mol^{-1} (r=0.970). The E_a value of 21 kcal.mol^{-1} for PPO inactivation indicates a high resistance of taro PPO to temperature increases. The E_a value of 19.4 kcal.mol^{-1}

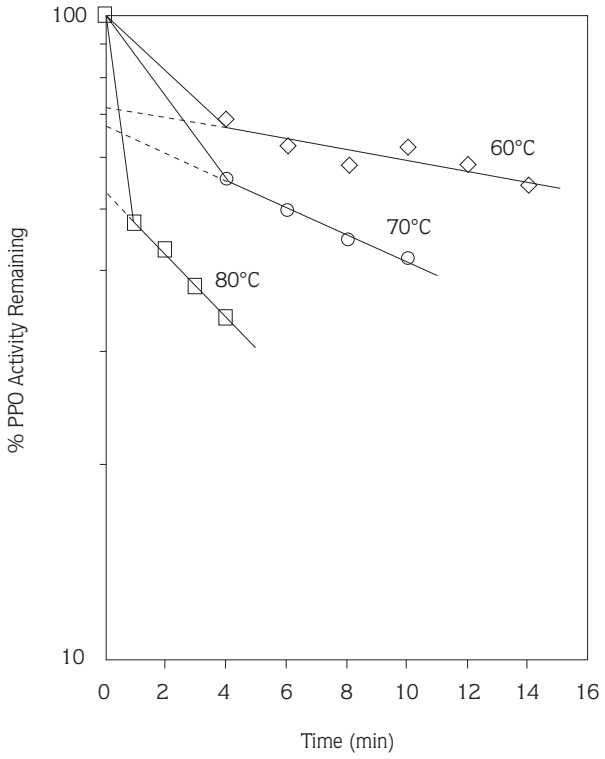


Figure 1. Heat inactivation of taro PPO in 0.05 M Na–phosphate buffer, pH 6.8.

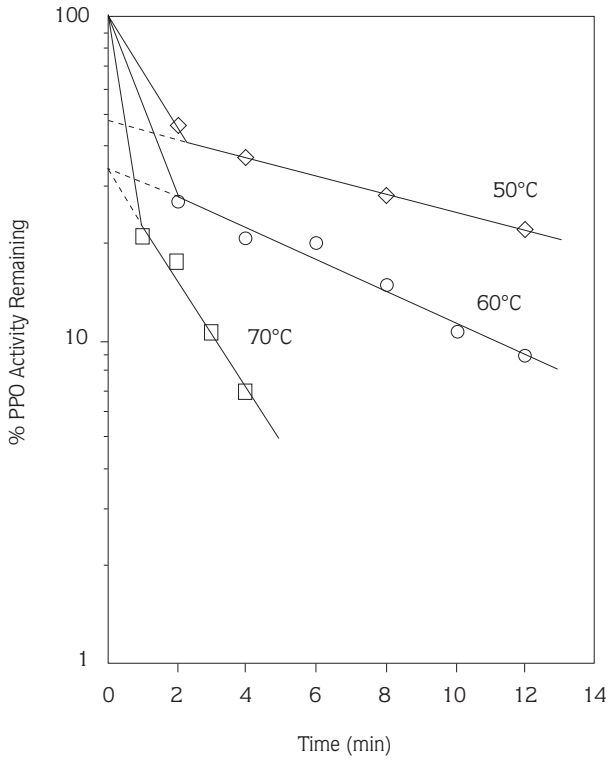


Figure 2. Heat inactivation of taro POD in 0.05 M Na–phosphate buffer, pH 6.8.

Table 1. Heat inactivation parameters of PPO and POD from taro in 0.05 M Na–phosphate buffer, pH 6.8.

	PPO			POD		
Temperature (°C)	Heat stable isoenzyme, (%)	D value (min)	$t_{1/2}$ (min)	Heat stable isoenzyme, (%)	D value (min)	$t_{1/2}$ (min)
50	–	–	–	48	35.4	10.6
60	72	124	26.9	34	20.8	6.2
70	67	47.5	14.3	33	6.0	1.8
80	53	20.4	6.1	–	–	–

for POD inactivation compares well with the following E_a values of heat stable POD isoenzymes: 21.3 kcal.mol^{-1} for cauliflower (21), 21 kcal.mol^{-1} for horseradish (17) and 21 kcal.mol–1 for corn (18).

D values of taro POD and PPO (Table 1) were calculated from the reciprocal of the slope of the inactivation curves. The half–life, $t_{1/2}$, was determined from the following equation:

$t_{1/2} = ln\ 0.5/k$

Comparing the D and $t_{1/2}$ values of PPO from some materials, which were also inactivated in phosphate buffer at similar pH values, taro PPO was more heat stable than PPO from Yali pear [$t_{1/2}$=6 min; 70°C] (22), guava [$t_{1/2}$=1.1 min; 75°C] (23), Royal Ann cherries [$t_{1/2}$=8 min; 75°C) (24), apricot [no activity was left at 80°C for 10 min] (25), apple [$t_{1/2}$=6 min; 70°C] (26) and avocado [$t_{1/2}$=8 min; 70°C] (27). On the other hand, a D value of 6 min at 70°C for taro POD is an indication of the presence of a heat labile POD enzyme. It is interesting that the z and E_a values of taro POD and PPO were almost the same. This indicates that both enzymes were affected similarly by the temperature increases. However, D values of PPO enzyme were relatively high compared with the D values of POD, which is considered the most heat stable enzyme of vegetables. Therefore, we recommend PPO rather than POD as an indicator of blanching for corms to prevent an enzymic browning reaction. Similar to our data, Fujita et al. (2) investigated cabbage PPO and POD, and indicated the presence of much more heat stable PPO compared with POD from the same source.

Effect of pH

The pH optimum of taro POD for o–tolidine and PPO for catechol was 5.9 and 6.5, respectively (Figure 3). The original pH of corm was 6.2 and at this pH value PPO and POD maintained 92 and 80% of their original activity, respectively. The results revealed that the activity of POD enzyme decreased sharply above pH 6.2. In contrast, PPO enzyme maintained 90% of its original activity between pH 6.0 and 7.5

Location of enzymes

The whole surface and internal parts of the corm flesh were covered with small cavities and cracks. The analyses of test papers revealed that both POD and PPO were present in these cavities and cracks. The formation of dense colors on the test papers indicated that PPO activity was condensed in the center of the corms. In contrast, POD activity occurred mainly under the skin with a little activity towards the center of the corms.

Substrate specificity of PPO

Taro PPO did not oxidize the phenol, a monohydroxy compound, but it did oxidize another monohydroxylic

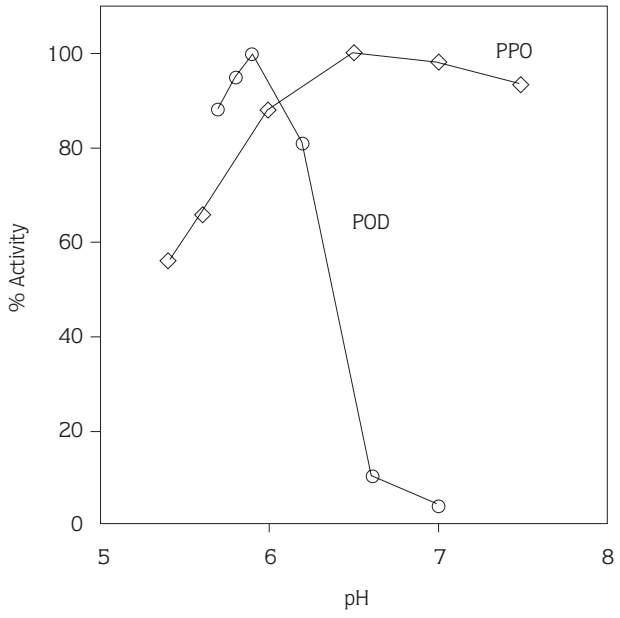


Figure 3. pH optimum for taro PPO and POD.

compound, p–cresol, with a lag period of 17 min. The oxidation of p–cresol indicates the presence of cresolase activity in corms. Moreover, hydroquinone, a p–dihydroxyphenolic compound, was not oxidized by taro PPO. This reveals that the enzyme laccase is not present in corms. The oxidation of phloroglucinol, a trihydroxyphenolic compound, occurred but this was not as rapid as that of catechol. As seen in Table 2, catechol, an o–dihydroxyphenolic compound, was oxidized at a much greater rate than the other phenolic compounds.

Inhibition of PPO

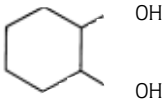
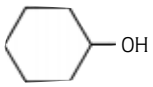
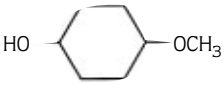
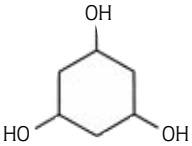
The effects of inhibitors on the activity of taro PPO are shown in Table 3. SO_2 and ascorbic acid at low concentrations effectively inhibited PPO activity. With EDTA, much higher concentrations (3.1–100 mM) were required to inhibit approximately 30% of PPO activity. In contrast, NaCl activated taro PPO at 103–651 mM concentrations. Only NaCl at an extremely high concentration (5 M) inhibited PPO slightly (19%). Ascorbic acid at 0.22, 0.35 and 0.40 mM concentrations caused a lag period of 0.5, 3 and 4 min in PPO activity, respectively.

Table 3. Effects of various inhibitors on the activity of taro ppo.

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	100.0	32.0
	50.0	31.0
	12.5	31.0
	6.3	33.0
	3.1	29.0
SO_2	0.60	100.0
	0.40	32.0
	0.34	21.0
	0.24	15.0
	0.08	3.0
NaCl	5000.0	19.0
	651.0	(8.0)*
	428.0	(48.0)
	216.0	(26.0)
	103.0	(8.0)
Ascorbic	0.80	100.0
acid	0.40	71.0
	0.35	54.0
	0.22	37.0
	0.06	6.0

* Number in brackets represents percentage increase in activity.

Table 2. Substrate specificity of taro PPO.

Substrate (0.1 M)	Formula	Wavelength (nm)	Activity ($\Delta_{OD}.min^{-1}.mL^{-1}$)	Catechol oxidation (%)
Catechol		420	0.864	100
Phenol		420	–	–
Hydroquinone		420	–	–
p–cresol		400	0.000643	0.077
Phloroglucinol		272	0.0168	1.94

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