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Kinetics of Thermal Inactivation of Peroxidases and Polyphenol Oxidase in Pineapple (*Ananas comosus*)

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Abstract The heat tolerance and the inactivation kinetics of peroxidase (POD) and polyphenol oxidase (PPO) in pineapples (*Ananas comosus)* were studied in the temperature range 45-95°C. The kinetic parameters, such as deactivation rate constant (k), activation energy (E_a), and decimal reduction rate (D) of the thermal inactivation process, were determined. POD in pineapples showed biphasic inactivation behavior at temperatures range 45-75°C but was monophasic at 85-95°C. This indicate that POD has 2 isozymes, namely heat labile and heat resistant, with E_a of 68.79 and 93.23 kJ/mol, respectively. On the other hand, the heat denaturation of pineapple PPO could be described as simple monophasic first-order behavior with E_a of 80.15 kJ/mol. Thus, the results of this study is useful in blanching technology where it shows a shortened time with higher temperature can be applied. The determination of the heat tolerance and inactivation POD and PPO, at different temperature range as done in the present work, was very important to improve the blanching process. This also will help to optimize the pineapple canning process which is one of the most important food industries in many tropical regions.

Keywords: *Ananas comosus*, pineapple, peroxidase, polyphenol oxidase, enzyme kinetics

Introduction

Pineapple, *Anana comosus*, is a perennial tropical moncot herb of Bromeliaceae and is one of the commercially important fruit constituting a major export item for many tropical countries. Beside the high sugar content up to 15%, the pineapple fruit is also considered as potential source for vitamins A, B, and C and many essential minerals. The fruits are either eaten fresh or processed and stored in form of slices or juice in cans and bottles. During the process of canning, large portions of fruits are sorted out and discarded. When the fresh pineapple fruits arrived in the canning factory, the fruits will be graded into several sizes according to the fruit's diameter. The fruit will be peeled thereafter, core removed, sliced, sorted, and canned. However during the process of canning, there are a big portion of fruits being wasted. This is particularly due to low availability of material recovery and reliable and economic process that can preserve the recovered portions of the fruits. Therefore, if blanching can be used to preserve this fruits and store them while demand arrive, it will save the production cost. Further more, these semi-processed fruits can be used to produce jam, juice, and assent based on market demand or to send it in its semi-processed form to other non pineapple producing country. Therefore, methods for preserving pineapple and prolonging its shelf-life are important. However, the presence of some enzymes in the fresh fruit ease its deterioration and leads to significant reduction in fruit quality through the change in texture, color, flavor, and nutritional value during storage (1,2). Of different enzymes responsible of fruit deterioration, peroxidases, catalases, polyphenol oxidases, and proteases are the most common biocatalyst governing this process.

Peroxidase (POD: E.C. 1.11.17) is an enzyme commonly found in fresh vegetables and fruits. It acts in hydrogen peroxide as an electron acceptor and oxidize a multitude of donor compounds. POD in foods cause the formation of undesirable end products unless they are completely or partially deactivated (3). It is also regarded as one of the most heat resistant enzymes (4,5) Therefore, inactivation of POD in food product can indirectly indicate that other enzymes are likely to be inactivated. In case of pineapple fruit, the black heart browning during storage is related to the enzymatic activity of POD (6). This internal damage has largely restricted the preservation of fruits for export. Beside POD, polyphenol oxidase (PPO) is also responsible for browning and off-color development in pineapple. PPO (E.C.1.10.3.1) is a copper containing enzyme which has the ability to convert *O*-dihyroxyphenol to *O*-benzoquinones, using oxygen as the secondary substrate that subsequently polymerizes nonenzymatically to brown pigments. This browning process leads to undesirable changes in color, flavor, and thus reflect negatively on the nutritional quality of the fruits. It is estimated that up to 50% loss of fresh tropical fruits are due to the bioactivity of PPO (7).

Of different methods applied for food processing, heat treatment is commonly applied to increase food stability during storage. Heat act as irreversible inactivator for both microorganisms and enzymes. Depending on the type of product desired, heat can be used to create varying degrees of preservation. Some examples of heat treatment applied to foods are sterilization, commercial sterility, pasteurization, and blanching. In general, blanching is applied until all the enzymes are completely deactivated. The factors which influence the final quality of the product are the adequacy

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of heat treatment and the method of treatment used (8). Factors which affect the adequacy of heat treatment are related to the extent of enzyme inactivation. In the present work, the heat inactivation profile for POD and PPO in pineapples was investigated. The thermal stabilities of POD and PPO in pineapples were also compared.

Materials and Methods

Plant material The pineapples fruits (*Ananas comosus* L. *Merryl*) used in this study were obtained from Simpang Renggam Plantation, Johor State, Malaysia. The fruits were washed and peeled. After removal of core, the fruit was cut into small pieces and stored at 4°C until used for experiments.

Chemicals Phosphate buffer solution, polyvinylpolypyrrolidone (PVPP), hydrogen peroxide (H_2O_2 1.5%), sodium chloride, and disodium hydrogen phosphate were obtained from Merck (Merck KGaA, Darmstadt, Germany). *p*-Phenylenediamine (PPD), catechol, and citric acid were obtained from Sigma-Aldrich (St. Louis MO, USA). All chemicals were of analytical grade.

Preparation of homogenates The refrigerated flesh was cut into small pieces, homogenized, and using fruit juicer (M3-70M; National Co., Tokyo, Japan). The obtained extract was quickly held on ice to prevent any change in enzyme content (9). These aliquots were prepared fresh before carrying out the experiments. This step reduces the fruit size and thus increases the heat transfer rate from the heating medium towards the tissue. By applying this method, the enzyme inactivation rate was based on the residual percentage without taking into account the size and structure of the fruits.

Thermal inactivation One mL aliquots of the pineapple homogenates were transferred to test tubes (Pyrex, 12 mm i.d.×150 mm) and held on ice until heating. Samples were heated in a circulated water bath (WNG 22; Memmert, Schwabach, Germany) to the indicated temperatures from 45-95°C for different specified time intervals between 0.5 and 15 min. After incubation at different temperatures for the desired time, samples were immediately cooled in ice bath. Before assay, the samples were warmed immediately at 25°C.

Peroxidase assay POD activity was assayed by measuring the rate of increase in absorbance at 485 nm ($\Delta Abs_{485\ nm}$) at room temperature using a UV-Vis spectrophotometer, (Genesis 10UV; Shimadzu Co., Tokyo, Japan), (10). The enzyme extraction was carried out by mixing 1 mL aliquot of the blanched homogenates with 5 mL of 0.05 M phosphate buffer (pH 7.0) containing 10 g/L of insoluble PPD. After this step, 25 μL of enzyme extract was mixed with 2.7 mL of 0.05 M phosphate buffer (pH 7.0), 200 μL of 10 g/L PPD and 100 μL of H_2O_2 (1.5% solution H_2O_2). The changes in absorbance of this mixture at 485 nm were measured every 15 sec up to 3 min. The enzyme activity was calculated based on the linear portion of the plot of $\Delta Abs_{485\ nm}$ against time (up to 3 min). A blank which consisted of 2.7 mL of 0.05 M phosphate buffer (pH 7.0), 200 μL of 10 g/L PPD and 100 μL of 15 mL/L of H_2O_2 was measured and the enzyme activities were deducted from this blank. One unit of POD activity was expressed as the change in absorbance at 485 nm/min and mL of enzyme extract.

Polyphenol oxidase assay PPO activity was determined spectrophotometrically at 410 nm based on standard method recently developed (11). One mL aliquot of the blanched homogenates was mixed with 1 mL of McIlvein buffer (pH 6.5) containing 5%(v/v) PVPP. The homogenate then was centrifuged at 34,020×g and 4°C for 30 min (Micro Centrifuge 22R; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). The solid residue was discarded and the supernatant was filtered through a Whatman No. 1 paper. After this step, 75 μL of supernatant (enzyme extract) was taken and mixed with 3 mL of 0.05 M catechol and the mixture was assayed. Absorbance readings were taken every 15 sec up to 3 min. This measurement was done against blank which consisted of 3 mL of catechol without enzyme mixture. The enzyme activity was calculated based on the linear portion of the plot of $\Delta Abs_{410\ nm}$ against time. One unit of PPO activity was defined as a change in absorbance at 410 nm/min and mL of enzyme extract.

Enzyme activity calculation Enzyme activity was determined by taking the slope of the plot of ΔAbs against time. From here, percentage of residual enzyme activities was calculated by taking the ratio of blanched and unblanched aliquots:

$$\text{Residual activity (\%)}=\frac{E}{E_0}\times 100 \quad (1)$$

where E is the activity of blanched aliquots and E_0 is the activity of unblanched aliquots.

The deactivation rate constant, k was calculated in 1/min by taking the slope of ln % residual activity vs. blanching time for each temperature 45-95°C.

$$\mathrm{Ln}\frac{[E]}{[E]_0}=-kt \quad (2)$$

The relationship between rate and temperature is described by the Arrhenius equation (Eq. 3) where R is the gas constant (8.314 J/mol/K), T is temperature in K. From the calculated k, a graph of ln k vs. 1/T was plotted. This is known as Arrhenius plot. The value for E_a was obtained from the slope of the plot.

$$\mathrm{Ln}\ k=-\frac{E_a}{RT}+\ln C \quad (3)$$

After obtaining the deactivation rate constant, k, the D-value (which is the reciprocal of k) was calculated as follows:

$$D=\frac{1}{k} \quad (4)$$

Results and Discussion

Residual activities of POD and PPO Figure 1 demonstrates the POD and PPO residual activities in aliquots of pineapple homogenates during the heat treatment at temperature ranging from 45 to 95°C for 15

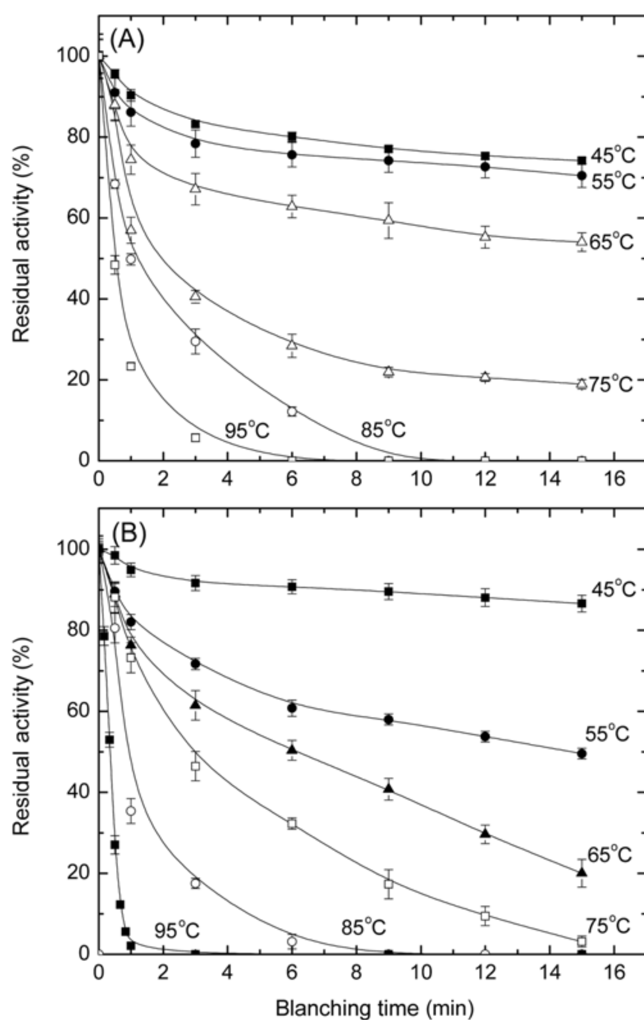

Fig. 1. Percentage enzyme residual activity vs. blanching time for pineapple. (A) Peroxidase (POD); (B) polyphenol oxidase (PPO). Data are the average taken from 3 experiments. The standard error based on these 3 values was calculated and expressed on the error bars.

min. In general, for both of POD and PPO the degree of enzyme inactivation was increased by the increase of both of temperature and exposure time. At all applied temperatures under study, the percentage of residual activities of POD were higher than those percentages obtained for PPO.

As shown in Fig. 1A, complete inactivation of POD in pineapple was achieved after 9 min of blanching at 85ºC. This inactivation time was reduced to only 6 min when the blanching temperature increased up to 95ºC. However, blanching at lower temperature (less than 75ºC) showed higher percentage of residual enzyme activity. After blanching for 15 min at 65 and 75ºC, the residual POD activities were approximately 54 and 22%, respectively. Whereas, on reducing the temperature to 45 and 55ºC, the residual activities of POD were about 74 and 70%, respectively.

For PPO in pineapple, complete inactivation was achieved in shorter time compared to POD inactivation (Fig. 1B). At 85 and 95ºC, PPO was inactivated after 6 and 3 min of blanching, respectively. Blanching at lower temperature (less than 75ºC) also showed lower residual activity of PPO compared to POD. At 65 and 75ºC, the residual PPO activity was 20 and 3% while at 45 and 55ºC, the residual activity was about 86 and 50%, respectively.

Figure 1 shows that PPO in pineapples was less heat stable than POD. At any temperature, POD showed higher percentage of residual activity compared to PPO. For example, when POD was blanched for 12 min at 75ºC, the enzyme loses 80% of its initial activity, whereas PPO loses 83% of its activity when heated for only 9 min at the same blanching conditions. This shows that POD required a longer heat treatment time compared to PPO to reach the same enzyme inactivation level at the same temperature. It has been also reported that POD in potatoes was more heat stable than other enzymes such as: PPO, lipoxygenase, and lipase (12). The higher thermal stability of POD compared to PPO in potatoes was also reported (1). On the other hand, in other study PPO was more heat resistant than POD in green coconut water and different types of vegetables (13). Thus, we can conclude that the thermal stability of POD and PPO is highly influenced by its origin and plant type specific.

Thermal inactivation curves for POD and PPO

***Peroxidase (POD)*:** The thermal inactivation curves, which demonstrate the change in ln % residual activity of the enzyme at different temperatures against the blanching time for POD in pineapple, are represented in Fig. 2A. As shown, 2 linear sections in curves were observed for the temperature between 45 and 75ºC. For these temperatures, POD pineapple was rapidly inactivated in the early period (0-3 min) of blanching and the inactivation rates were slowed down thereafter. As shown, the inactivation of POD in pineapple did not follow first order for temperature between 45 and 75°C. The biphasic inactivation curve shows 2 linear fractions with different inactivation rate for each temperature. The first part was due to the presence of heat labile POD which was rapidly inactivated rapid inactivation process, whereas the second part of slower inactivation rate was due to the presence of less heat sensitive enzyme fraction. On the other hand, blanching at higher temperature between 85 and 95ºC, POD inactivation followed monophasic first-order kinetic.

This biphasic pattern for enzyme inactivation was also reported for POD of carrot when thermally treated at temperature range 45-65ºC (27). They assumed that this behavior was due to the presence of heat labile and heat resistant enzyme fractions. They have been observed that enzyme inactivation was following monophasic first order kinetic at higher temperature. Other thermal inactivation studies of POD in different fruits and vegetables showed also biphasic curves. Distinguished biphasic inactivation behavior of POD in watercress was observed also when the enzyme thermally treated at temperature range 40-92.5ºC (14). It has been also reported that inactivation of asparagus POD followed biphasic patterns in temperature range from 50 up to 70ºC (15). This biphasic inactivation behavior of POD in corn was also observed when enzyme inactivated at high temperature between 82 and 99ºC (16). Other study of revealed also that the thermal inactivation process of POD in beans, asparagus, zucchini, and carrots are also following biphasic inactivation kinetics (3). Contrary to the

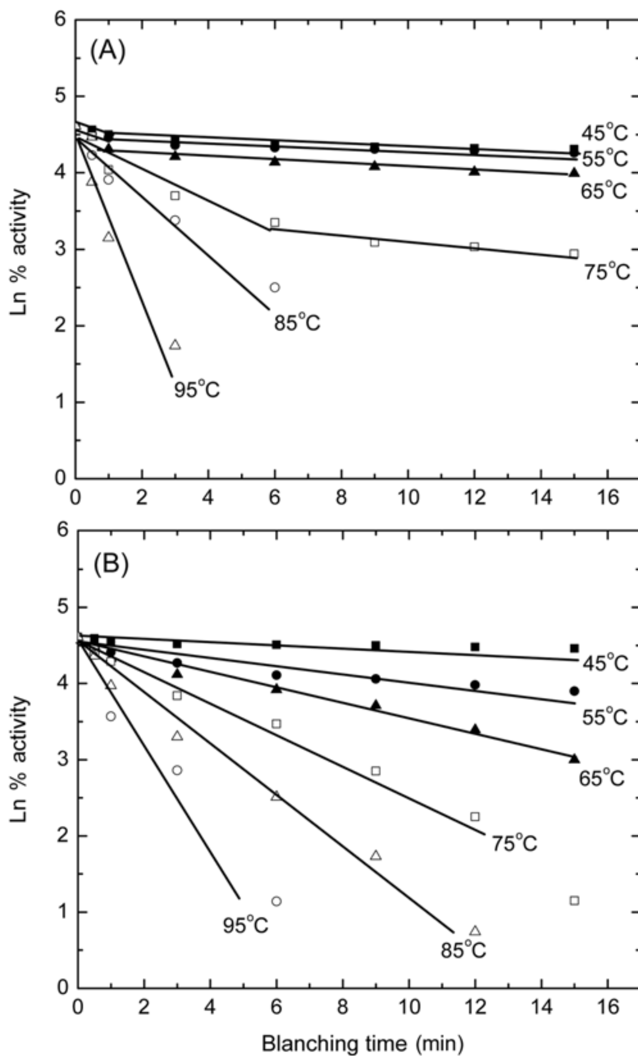

Fig. 2. Ln % residual activity vs. blanching time for in pineapple. (A) Peroxidase (POD), (B) polyphenol oxidase (PPO).

result that the inactivation of POD follows biphasic curve, some other authors reported first order inactivation kinetics in carrots, potatoes, and horseradish (1,17,18).

In fact, there were 2 major arguments regarding the biphasic curves obtained for POD. The first argument is that there are 2 iso-enzymes of POD with different heat stabilities, and the second is that a protective mechanism is probably operating (19). POD is known to exist in iso-enzymes which can be divided into 2 distinct groups according to their physical and catalytic properties. Therefore, the argument on that biphasic inactivation in POD is due to the iso-enzymes is generally accepted. The POD in asparagus was found also to follow biphasic behavior when thermally inactivated at 70°C. This was attributed to the presence of iso-enzymes with different heat stabilities (15). However, some studies have shown that even isolated enzyme from POD still showed biphasic inactivation curve, which means that the presence of iso-enzymes could not easily explain the kinetics of inactivation (19,20). Instead, different physical states of POD must be taken into account to explain the biphasic curve. However, in this study, heat treatment was applied to POD in aliquots of homogenates and hence there should be no difference in the physical states of POD.

***Polyphenol oxidase (PPO)*:** The plot of ln % residual activity of PPO vs. blanching time at different temperature is shown in Fig. 2B. This enzyme show simple first-order inactivation kinetics with no evidence of multiple isozymes. This result is in agreement with many other authors who also obtained first-order kinetics for thermal inactivation of pineapple puree, apple cultivars, table beets, and d'Ajou pears (21-24).

***Enzyme inactivation kinetic parameters*:** The following kinetic inactivation parameters, the deactivation rate constant k; decimal reduction value D, and activation energy E_a were determined for POD and PPO in pineapple.

***k and decimal reduction time (D-value)*:** Two parameters that can be used to indicate the thermal stability of enzymes are the rate constant, k and D-value, which is the time required to reduce 90% of enzyme activity (25,26). For POD in pineapples, the inactivation was resolved to heat labile and heat resistant fractions. Therefore, 2 deactivation rate constants had to be determined for each temperature. The k and D-value for heat labile and heat resistant POD are shown in Table 1. It is found that k of heat labile fractions were higher than k of heat resistant fractions whereas it is the opposite for D-value. Higher k or lower D-value indicates higher rates of inactivation. This shows that the heat labile fractions were inactivated faster than heat resistant fraction for the thermal inactivation of POD in pineapple and are less heat stable compared to the heat resistant fractions of POD.

Table 2 shows the k and D-value for thermal inactivation of PPO in pineapples. Referring to Table 1 and 2, and comparing only heat resistant fractions of POD with PPO, the value of k and D depended on temperature. At all temperature PPO was more stable than POD except 75ºC where POD was only slightly more heat stable than PPO.

Activation energy (E_a) Activation energy, E_a, for thermal

Table 1. k and D-values of thermal inactivation of peroxidase (POD) in pineapple

Temperature (°C)	k (1/min)		D (min)	
	Heat labile	Heat resistant	Heat labile	Heat resistant
45	0.0587±0.0101	0.0097±0.0012	17.05±2.92	103.26±11.23
55	0.1495±0.0221	0.0122±0.0005	6.69±0.99	82.04±16.69
65	0.2952±0.0194	0.0222±0.0016	3.39±0.22	45.14±4.99
75	0.5625±0.1777	0.0768±0.0125	1.78±0.56	13.02±2.23
85	N/A	0.3282±0.0319	N/A	3.05±0.30
95	N/A	0.9139±0.1250	N/A	1.09±0.15

Table 2. k and D-values of thermal inactivation of polyphenol oxidase (PPO) in pineapple

Temperature (°C)	k (1/min)	D (min)
45	0.0035±0.0003	286.58±23.61
55	0.0046±0.0004	216.28±20.12
65	0.0203±0.0009	49.38±2.16
75	0.0960±0.0045	10.42±0.48
85	0.1441±0.0077	6.94±0.37
95	0.3814±0.0159	2.62±0.11

Table 3. Activation energies (E_a) for peroxidase (POD) heat labile and heat resistant fractions in other fruits and vegetables

Fruits/Vegetables	E_a (kJ/mol)		Reference
	Heat labile	Heat resistant	
Carrots	89.6	148	27
Carrots	52	57	30
Carrot (cortex)	95	86	28
Peas	41	75	30
Green beans	57	77	30
Potato	83	104	31
Broccoli	75	58	28
Asparagus (stem)	61	53	28
Horseradish	142	88	32
Watercress	421	352	14
Pineapple	68.98	93.23	This work

inactivation of POD in pineapple was calculated from Arrhenius plot. The activation energies for heat labile and heat resistant fraction of POD are as shown in Table 3. Referring to Table 3, the values of E_a from this study was in agreement with those obtained by other researchers (27), in which the E_a is higher in heat resistant fraction of POD compared to heat labile fraction. It have been also reported that heat resistant fraction of pectin methyl esterase (PME) in carrots and potatoes have higher E_a compared to the heat labile fraction (1). However, there are some findings that contradict with this result. However, since POD inactivation in pineapples was not studied by other authors, therefore the obtained E_a in this study was compared with other fruits and vegetables (Table 3). In this study, the E_a of heat resistant fraction of POD was higher than those obtained for heat labile fraction of POD.

Activation energies, E_a for thermal inactivation of PPO in pineapples were calculated from Arrhenius plot. The Arrhenius plot for PPO in pineapple shows a simple linear fit with a slope equivalent to E_a of 80.15±6.69 kJ/mol. This means that PPO in pineapple is very heat resistance compared to POD, and this agrees with the value of k in Table 1 and 2. However, Table 4 summarizes a simple comparison between the E_a values of PPO of different types of fruits and vegetables. The values of E_a reported for POD and PPO in Table 3 and 4 vary quite considerably. These differences might be heat transfer related issues arising from differences in fruit preparation method and containers used for thermal inactivation of enzymes. For example: some research group used carrot as the source of POD enzyme for testing and the preparation steps to

Table 4. Activation energies (E_a) for polyphenol oxidase (PPO)

Fruits/Vegetables	E_a (kJ/mol)	Reference
Amasya apple	0.11841 (15-80°C)	33
Victoria grape	225 (55-65°C)	34
Apple	55.1-77.2 (68-78°C)	22
Pineapple (puree)	23.7 (40-70°C) 82.8 (70-90°C)	21
Pineapple (aliquot)	80.15 (45-95°C)	This work

prepare juice extract include the use of blender and addition of water followed by centrifugation to remove the solid content (27). Only the supernatant was used for thermal inactivation, whereas, other groups use the complete extract for enzyme assay (28). Furthermore, the containers for thermal inactivation of enzyme were also not standardized. Researchers used fresh vegetables, capillary tubes, and test tubes for the thermal inactivation process. This caused differences in the heat transfer rate during the thermal inactivation due to the huge differences in heat contact surface in the reaction system.

Apart from the heat transfer, the enzyme and assay method could also caused differences in the results obtained. The hydrogen donors that were commonly used for the enzyme assay were *O*-dianisidine, 3-ethlubenzotiazol-6-sulphoric, guaicol, pyrogallol, and 3-ethylbenzthiazoline-6-sulfonic acid (for POD). Other authors used titrimetric method based on the oxidation of ascorbic acid for enzymatic assay (29). However, it has been also observed that the stabilities of isoenzymes varies with the donor substrate supplied, therefore the choice of the donor was important for the inactivation experiments (27).

Kinetic parameters can be calculated using 3 different least-square methods: 2-step linear regression, 1-step linear regression, and nonlinear regression. In the study of Rodrigo *et al.* (4), they obtained the results using 3 different procedures and showed that there was a greater similarity between the kinetic parameters estimated by 2-step linear regression and by nonlinear regression than those calculated by 1-step linear regression (4). They also pointed out that the asymptotic standard deviation were generally greater with 2-step linear regression.

In case of PPO and using pineapple as the polyphenoloxidase source for thermal inactivation experiment, the obtained E_a value was 23.7 kJ/mol of enzyme blanched between 40-70 (21). The value of E_a was increased up to 82.8 kJ/mol when the applied temperature was in the range of 70-90ºC. Those values are close of those obtained in our study (E_a=80.15 kJ/mol for the temperature range 45-95ºC). However, no further detail was explained by the named author on why they separate E_a into 2 section which is 40-70ºC and 70-90ºC. In our study the enzyme inactivation in case of PPO was first order reaction. However, the E_a value that obtained by them should have a lower value as assumption was made when the 2 values are being average out [(23.7+82.8)/2=53.25 kJ/mol]. The differences between our obtained results in the present work and these results may due to the material of the experiment used was different from what was used in this test. They used a sample in form of fruit pulp and the sample used was not freshly prepared, these 2 factors may reduce the PPO

activity since the heat transfer rate in homogenized sample is different of those of fruit pulp and the PPO activity may decreased in non-freshly prepared samples due to enzyme degradation.

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