ACTIVITY OF PEROXIDASE (POD) EXTRACTED FROM RIPE PAWPAW (*Carica papaya*) FRUIT PULP

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Abstract: Crude peroxidase (POD) was extracted from ripe pawpaw (*Carica papaya)* fruit pulp and the enzyme kinetics, activity at varying pH and temperature conditions were studied. The enzyme exhibited maximum activity at pH 7.0 at 10-20^{o}C. The enzyme lost 60% activity at 70^{o}C. The POD had reaction constant (K_m) of 0.113M and a maximum catalysis rate (V_{max}) of 0.013 enzyme units/g fruit pulp. The enzyme was completely inactivated when incubated at 90^{o}C. The result of the study suggested that crude peroxidase from ripe pawpaw fruit pulp was stable over wide temperature ranges. The value of K_m suggests that POD from ripe *Carica papaya* fruit pulp can be isolated, purified and characterized.

Keywords: Enzyme, inactivation, ripe pawpaw, peroxidase, pH, temperature .

Introduction

The tropics with their abundant moisture and warm to hot temperatures produce the most diverse and abundant fruits such as pawpaw (*Carica papaya*). Pawpaw (*Carica papaya*) is a member of the Caricaceae family which if wild grows to about 1.8 m high, but the cultivated once may be about 7.6 m high (Kingsley, 2009). The shape of the fruit varies from spherical to elongate and may weigh as much as 9 kg. Pawpaw fruit is highly nutritious when compared with other fruits like apple, banana and orange (Grant, 2008). The fruit has high protein content, carbohydrate, vitamins (A and C), minerals and essential acids (Peterson *et al.*, 1982). Pawpaw fruit is climacteric, which means it may be picked when mature before ripening has commenced and subsequently ripened postharvest. Indicators of ripening include changes in the colour of the flesh from green to yellow, softening of the flesh, increased production of soluble solids and increased volatile flavor (Achbold & Pomper, 2003). Pawpaw fruit can be eaten fresh or used in salad, deserts or in value added products such as ice creams, yogurts and baked foods (Pomper & Layne, 2005).

Consumers of fruits and vegetables consider the quality of fruits in terms of colour, flavor, texture and nutritional value before selection is made. To respond to this selection, food processors make considerable effort to improve the quality of fruit and vegetables from the harvest through storage to the end of processing. Endogenous enzyme such as peroxidase (POD) can have profound effect on the quality of fruits and vegetables (Queiro *et al.*, 2008). Peroxidase (POD) is an oxidoreductase enzyme which catalyzes the reduction of peroxides and the oxidation of a variety of organic and inorganic compounds. It is found in many plant based foods (Ziaebrahim *et al.*, 2007) and play roles in food quality, including deteriorations of colour and flavor (Benjawen *et al.*, 2006).

The enzyme POD is also found in the tissue of a wide range of fruits and vegetables and is responsible for the brown colouration observed in these food materials (Fang, 2007). Valderama & Clemese (2004) also observed that the peroxidase enzyme family is involved in a great number of oxidative reactions such as colour change, chlorophyll degradation, phenol and indole acetic acid oxidation, lignin biosynthesis, flavour development, pulp softening and food quality modification. Enzymatic browning is very common at the cut surface of light-colour fruits (Ebiloma *et al.*, 2011). The cut surface may rapidly change to brown due to the oxidation of phenols to orthoquinones which quickly polymerise to form brown pigments or melanins (Ebiloma *et al.*, 2011).

Peroxidase activity has been studied in apple, carambola fruit and mango kernel by researchers (Leja *et al.*, 2003; Perez-Tello *et al.*, 2001; Ebiloma *et al.*, 2011). The objective of this study was to determine the activity of POD extracted from ripe pawpaw fruit pulp at varying pH and temperature conditions and to characterize its enzyme kinetics. The information obtained could be useful in the development of treatment technology and storage conditions that would control enzymatic browning.

Materials and methods

Collection of samples

Ripe pawpaw (*Carica papaya*) fruits were harvested from Enugu state of Nigeria. The epicarp of the fruits was removed and the fruit cavities cleaned. The fruits were then sliced horizontally into halves with a sharp knife and the sliced fruit pulps were taken to the Medicinal Department of National Institute of Pharmaceutical Research and Development (NIPRED) Idu, FCT, Abuja for further treatment.

Preparation of samples

The sliced fruit pulps were weighed, homogenized using a domestic blender, filtered using a cheese cloth and freeze-dried. The freeze-dried sample was stored in an air tight sample bottle and used for analysis.

Crude POD enzyme extraction

Crude POD enzyme was extracted using the method described by Ebiloma *et al*. (2011) and Anjumzia *et al*. (2011). The freeze-dried fruit pulp sample (1.0 g) was mixed with 1.5 ml prechilled 0.01M sodium phosphate buffer (pH 6.0). Each mixture was stirred at room temperature for 5 min to extract POD. This was then centrifuged at 10000 rpm for 15 min at 4^{o}C to remove all debris. The supernatant was carefully removed from the sediments and filtered using Whatman No.1 filter paper.

Determination of POD enzyme activity

The procedure for determination of peroxidase enzyme activity using pyrogallol as the substrate as described by Fang (2007) and Ebiloma *et al.* (2011) was adopted for the analysis. The crude POD extract was diluted 5 times with 0.01 M sodium phosphate buffer (pH 6.5). A constant volume of 0.32 ml of 5 % pyrogallol was mixed with 0.4

ml of each categorized crude enzyme extract and 0.16 ml of 35 % hydrogen peroxide solution. A portion of each enzyme-substrate mixture was rapidly transferred into a cuvette and the absorbance measured at 420 nm for 60 s interval up to 360 s using a uv-spectrophotometer. Before taking absorbance readings, the instrument was zeroed using the same combination of reagents above but without the enzyme extract. Replicate readings were taken for the test. The average of the replicated readings was taken. Enzyme activity was calculated in enzyme unit/g fruit pulp using:

$$\text{Enzyme units/g fruit pulp} = \frac{\Delta Absorbance\,(420\ nm)\ x\ V_a\ x\ df}{\Delta Time(s)\ x\ 12V_e}$$

Where: V_a = Vol. of assay; df = Dilution factor; 12 = Extinction coefficient of 1.0 mg/ml of purpurogallin at 420 nm; V_e = Vol. of enzyme extract used

Evaluation of effect of pH

The effect of pH on POD activity was determined as described by Fang (2007). 0.4 ml of the diluted POD extract was mixed with 0.32 ml of 0.01M sodium phosphate buffer (pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5). The POD activity was tested as described earlier.

Evaluation of effect of temperature

The effect of temperature on POD activity was determined as described by Fang (2007). The diluted POD enzyme extract (0.4 ml) was mixed with 0.32 ml 0.01 M sodium phosphate buffer (pH 6.0) and was incubated for 10 min at varying temperatures ranging between 10 to 90 ^{o}C in an isotherm water bath. After incubation the mixture was cooled in ice slurry and the activity determined as described above at the various temperatures.

POD kinetics

The reaction rate of POD at a series of substrate concentration was determined as described by Ebiloma *et al.* (2011). Hydrogen peroxide (0.16 ml of 35 %) and a constant 0.4 ml of the diluted enzyme extract was mixed with 0.32 ml of 0.01M sodium phosphate buffer (pH 6.5) followed by the addition of 0.32 ml pyrogallol (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40M). The absorbance was measured immediately every 60 s interval up to 360 s. The absorbance versus time and the reaction rate (enzyme activity) versus substrate concentration were plotted to fit the Michalis- Menten equation and to derive the Michalis constant (K_m) and the maximum velocity (V_{max}) (Fang, 2007).

Statistical analysis

Data were expressed as means ± SD of three independent experiments. A nonlinear regression was used for enzyme kinetics.

Result and Discussion

There was no brown colour in the crude POD extract before use. POD activity was spectrophotometrically measured using pyrogallol as the substrate. Spectrophotometric measurement of peroxidase activity of fruits provides a quantitative basis for determining the amount of the enzyme present in the fruit (John, 1999). On addition of pyrogallol to the fruit pulp extract, brown colour was observed. The intensity of the characteristic brown colour produced during reaction expressed the amount of enzyme and the magnitude of enzyme reaction in the crude extract.

Fig. 1 shows the effect of pH on the activity of crude POD extracted from ripe pawpaw. The crude extract of pawpaw fruit pulp exhibited significant POD activity over a broad range of pH. The absorbance of all the fruit pulps extracts increased linearly within the initial 60 s displaying a first order enzymatic reaction behavior. The absorbance of the fruit pulp increased gradually within 120 to 240 assay time and reached a plateau after 240 s.

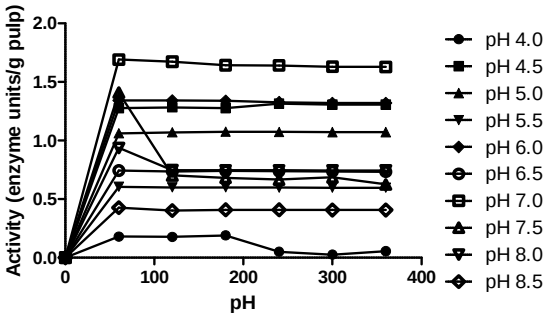

Fig. 1: Effect of pH on the activity (absorbance of crude POD extracted from ripe pawpaw fruit pulp) at room temperature.

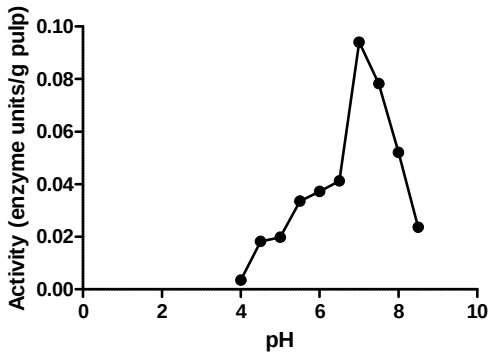

Fig. 2: The optimum pH for crude POD extracted from ripe pawpaw fruit pulp at room temperature.

The absorbance of POD activity on pH followed a bell-shaped curve (Fig. 2). The activity increased steadily from pH of 4.0 to 6.5; reached its peak at pH 7.0 and then decreased steadily from pH 7.5 to 8.5. This suggests that ripe pawpaw in POD was unstable at pH greater than 7.0 or that residual proteins present in the enzyme reacted with the reaction product (oxidized pyrogallol) to form quinone polymers (Fang, 2007). A maximum activity of 0.094 was at pH 7.0. This pH maximum coincided with the natural pH (6.9) of pawpaw fruit pulp (Fang, 2007). Fang (2007) observed that fruits susceptible to browning show high activity at pH around 6.5-7.0.

Figs. 3 and 4 show the result of the activity of crude POD extract after 10 min exposure to temperatures ranging from 10-90^{o}C. The enzyme extract showed highest activity (0.062 enzyme units/g fruit pulp) when incubated at 10^{o}C. At temperatures of 20^{o}C to 60^{o}C, the activity decreased slightly until 70^{o}C when the loss of activity became remarkable; 60% activity was lost. Activity was completely lost when the enzyme was incubated at 90^{o}C (negative). The result showed that crude POD extracted from ripe pawpaw fruit pulp was stable over wide temperature ranges. This finding is in consonance with that

of Reed (1995) who noted that peroxidase enzyme is the most heat resistant enzyme and is used as an index of blanching procedures. The high activity of POD at 10-20^{o}C indicated the reaction of the enzyme with the substrate (pyrogallol) while the reduced activity at high temperatures (70^{o}C) was probably due to thermally induced irreversible conformation (denaturation) changes in POD (Casado Vela *et al.*, 2006).

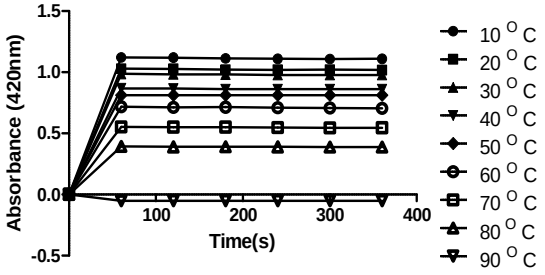

Fig. 3: Effect of heating on the activity (absorbance) of crude POD extracted from ripe pawpaw fruit pulp at 25^{o}C.

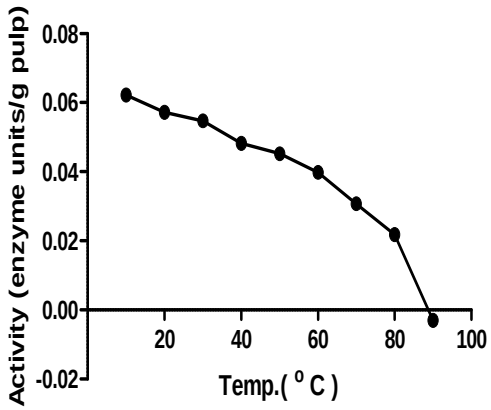

Fig. 4: Influence of heating on the activity (absorbance) of crude POD extracted from ripe pawpaw fruit pulp at 25^{o}C.

The POD enzyme kinetics was determined with substrate concentration ranging from 0.05 to 0.40M. The plot of absorbance against substrate (pyrogallol) concentration (Fig. 5) showed that the absorbance increased linearly within the first 60 s of reaction time and a plateau was observed between 120-240 s. There was corresponding increase in POD activity as the substrate concentration increased (Fig. 6). This indicates that addition of more of the substrate would increase the reaction rate and that the maximum reaction rate that can be achieved by a certain enzyme level, would be the reaction rate at the saturation point. The results of activity (reaction rate) and substrate concentration were subjected to non-linear regression analysis to generate the Michaelis-Menten plot from where K_m and V_{max} were obtained.

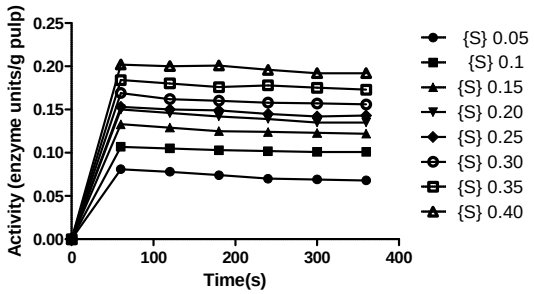

Fig. 5: Effect of substrate concentration on enzyme activity (absorbance of crude POD extracted from ripe pawpaw fruit pulp) at 25 ^{o}C.

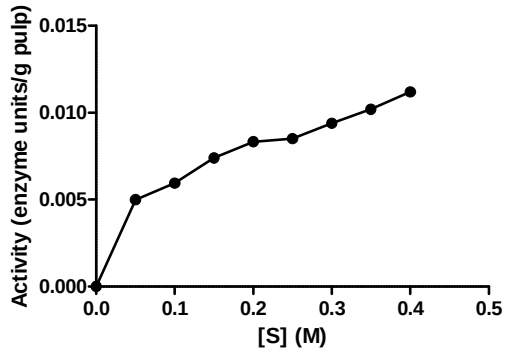

Fig. 6: Michalis-Menteu plot of reaction rate (activity versus substrate (pyrogallol) concentration for crude POD) extracted from ripe pawpaw fruit pulp at 25^{o}C.

The curve (Fig. 6) was not sigmoidal in shape; therefore, POD enzyme from ripe pawpaw fruit pulp cannot be described as allosteric. K_m and V_{max} are 0.113M and 0.013 enzyme units/g fruit pulp, respectively. The reaction constant (K_m) which is the Michaelis-Menten constant is a measure of the affinity between an enzyme and its substrate and describes the rate of dissociation of the enzyme-substrate complex (ES). Large K_m value represents low affinity and weak enzyme-substrate association whereas low K_m indicates a strong enzyme-substrate complex and high affinity (Ebiloma *et al*., 2011). Yu *et al.* (1994) reported K_m of 58 µM and V_{max} of 3.36 units/mmol for French bean peroxide using pyrogallol as substrate; Shukla *et al*. (2004) also reported K_m of 8×10^{-5} and V_{max} of 1.53 for mango (*M. indica*) peroxidase when hydroquinone was used as substrate while Ebiloma *et al*. (2011) reported K_m of 1.48 mM and V_{max} of 0.29 enzyme units/g kernels for *M. indica* peroxidase using pyrogallol as substrate. These results suggest that K_m and V_{max} values for peroxidase activity may vary with the type of substrate, source, fruit specie, maturation and purity of the enzyme.

Conclusion

The results of the study showed that ripe *Carica papaya* contained significant peroxidase activity. It exhibited maximum activity at pH 7.0 and in the temperature range of 10^{o}C to 60^{o}C; at 90^{o}C it was completely inactivated. The enzyme was stable over wide temperature ranges and can be inactivated or inhibited by heating or pH adjustment (pH 4.0 to 8.5). The K_m of 0.113M using pyrogallol showed that POD in ripe pawpaw fruit pulp can be isolated, purified and characterized.

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