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Polyphenoloxidase from Starfruit (*Averrhoa carambola*, L.) [1]

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ABSTRAK

Polifenoloksidase (PPO) telah diasingkan daripada belimbing besi (Averrhoa carambola, *L.) dan ditulenkan melalui pemendakan aseton dan dialisis. pH optimum untuk pengoksidaan katekol yang dimangkinkan oleh PPO yang ditulenkan dengan aseton adalah 7.2. Kajian penyahaktifan haba menunjukkan bahawa enzim PPO adalah labil terhadap haba. Hayat-separa untuk penyahaktifan PPO pada 70°C dan 75°C masing-masing ialah 4.6 min dan 3.1 min. Didapati juga bahawa malar Michaelis (K_m) dan V_{max} untuk katekol dengan PPO masing-masing ialah 0.061M dan 20 U ml^{-1}.*

ABSTRACT

Polyphenoloxidase (PPO) was isolated from starfruit (Averrhoa carambola, *L.) and purified by acetone precipitation and dialysis. The optimum pH for the acetone purified PPO catalysed oxidation of catechol was found to be pH 7.2 Heat inactivation studies showed that the enzyme was heat-labile. Half-lives for PPO inactivation at 70°C and 75°C were found to be 4.6 min and 3.1 min respectively. The Michaelis constant (K_m) and V_{max} for catechol with PPO was 0.061M and 20 U ml^{-1} respectively.*

INTRODUCTION

Starfruit (*Averrhoa carambola,* L.) is considered to be under the oxalidaceae or oxalis family. Like most other fruits and vegetables, the starfruit discolours during storage or when it is cut and processed. The discolouration leading to browning is a consequence of non-enzymic as well as enzymic reactions involving polyphenolic compounds. The specific enzymes which take part in browning reactions have been referred to generally as polyphenoloxidases (Mayer and Harel, 1979; Vamos-Vigyazo, 1981). (Enzyme number 1.14.18.1 with the systematic name of monophenol, dihydroxyphenylalanine: oxygen oxidoreductase; Anon, 1973).

A considerable amount of work has been done on the isolation and characterization of polyphenoloxidase. It has generally been found that PPO is a relatively difficult enzyme to extract and purify (Mayer and Harel, 1979). Although there appears to be a wealth of information on the subject, the overall picture of polyphenoloxidase is not complete.

This is because (i) PPO acts on a great number of substrates and catalyses more than one reaction (ii) PPO can occur in multiple forms in individual species (iii) the physiological role of the enzyme is not understood and (iv) it has a complex role in food processing (Vamos-Vigyazo, 1981).

It is also clear that the characteristics of PPO vary with the type of fruit. There are differences between PPO from different cultivars

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and also differences in PPO isolated at different stages of maturity of fruits and vegetables. This has become evident from reports on PPO from peaches (Wong *et al.*, 1971; Luh and Phithakpol, 1972; Flurkey and Jen, 1978), banana (Galeazzi and Sgarbieri, 1981; Galeazzi *et al.*, 1981; Jayaraman *et al.*, 1982), cherries (Benjamin and Montgomery, 1973), guava (Augustin *et al.*, 1985), pears (Halim and Montgomery, 1978; Montgomery and Petropakis, 1980), avocado (Kahn, 1975; Kahn, 1977) and mango (Park *et al.*, 1980). The characteristics of PPO from a large variety of fruits and vegetables has been reviewed by Vamos-Vigyazo (1981).

There has been no work reported on starfruit PPO. An understanding of the enzyme will help efforts in controlling the undesirable browning of the fruit and processed starfruit products. The present work involved the extraction and purification of PPO from starfruit and a study of its enzymic activity using catechol as a substrate.

MATERIALS AND METHODS

Materials

Starfruits were obtained from the area around Universiti Pertanian Malaysia. The age of the fruit was estimated at 1.5 months. The fruits had a yellowish-green colour.

Extraction and Purification of PPO

The method used was based on that of Galeazzi *et al.*, (1981). 100 g of longitudinally sliced fruit was homogenized in a blender for 30 sec with 100 ml of 0.2M phosphate buffer (pH 6.8) containing 1.5% polyvinylpyrrolidone (PVP) and 0.5% Triton X-100. Homogenates were centrifuged at 12 000 rpm (25,500 g) for 15 min at 4°C (Du Pont RC-5B Centrifuge). The enzyme remained in the supernatant (crude extract).

Previous reports have shown that one of the major difficulties encountered during preparation of a soluble PPO is that of preventing enzymatic phenol oxidation and polymerization during extraction. This may cause irreversible inactivation of the enzyme and a resultant low activity of the extracted enzyme (Vamos-Vigyazo, 1981). In this work, PVP which is a phenol scavenger was added to the extraction buffer during the isolation step. This minimizes enzyme denaturation by phenol oxidation products (Stelzig *et al.*, 1972). Triton-X was incorporated into the extraction medium because it functions as a solubilization agent.

Purification of the crude extract involved acetone precipitation and dialysis. All purification steps were carried out at 4°C. The acetone precipitate was obtained by addition of 2 volumes of cold acetone and recentrifugation at 15 min at 12 000 rpm (25 500 g) at 4°C. The precipitate was reextracted with one volume of 0.2M phosphate buffer (pH 6.8). For the dialysis step, the reextracted solution containing the acetone-precipitated enzyme was put into a dialysis bag (Sigma Dialysis 'Sacks' 250-7U) and dialysed against 0.2M phosphate buffer (pH 6.8). The dialysis was carried out at 4°C for 48 hrs with four changes of dialysing media.

PPO Assay

Enzyme activity was determined by measuring the increase in absorbance at 410 nm. The reaction mixture contained 1 ml 0.05M catechol, varying amounts of enzyme extract and 0.2M phosphate buffer (pH 6.8) in a final volume of 5 ml. The temperature of the assay was 30°C. The rate of the reaction was calculated from the initial linear slopes of activity curves. One unit (U) of PPO activity was defined as the amount of the enzyme that increased the absorbance by 0.001 min^{-1} under the conditions of the assay (Benjamin and Montgomery, 1973; Galeazzi *et al.*, 1981).

The pH profile of the enzyme was determined in th range of pH 5.0 — pH 8.0 using 0.2M phosphate buffer and the conditions of the assay described above. Specific activity (U mg^{-1}) was plotted against pH to get the pH optimum.

The thermal denaturation of the enzyme (acetone-precipitated extract) was obtained by adding 3 ml of the enzyme extract in test-tubes and heating at 70°C, 75°C and 80°C respectively. After various heating intervals, 0.5 ml samples were withdrawn and immediately cooled in ice-water (Park *et al.*, 1980; Wissemann and Lee, 1981). It was then assayed for PPO activity with 0.05M catechol as the substrate at pH 6.8 under the conditions of the assay described above.

Determination of K_m and V_{max}

The Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined by varying the catechol concentrations of the reaction mixture over the range 0.02M – 0.10M catechol. The temperature and pH of the reaction mixture was 30°C and pH 6.8 respectively. The data was plotted as 1/ activity against 1/ catechol concentration.

Protein Determination

Protein concentration of the extracts was determined using the Lowry method as given by Layne (1957). Bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

Isolation and Purification of PPO

The results of the extraction and purification of starfruit PPO are given in Table 1. The crude extract had low specific activity. Purification by acetone precipitation and dialysis resulted in a 9.5 and 22 fold increase in the specific activity.

Acetone precipitation has often been used in extraction and purification steps for PPO. Flurkey and Jen (1978) found that activities of peach PPO were greatly enhanced by acetone powder preparation. It was suggested that the 20-fold increase in PPO activities in extracts of acetone powder over that of fresh extracts of peach PPO was due to the presence of inhibitory substances in fresh extracts or possibly because of aggregation of PPO isoenzyme forms during acetone powder preparations (Flurkey and Jen, 1978). The possibility of these factors coming into play in the starfruit PPO preparation cannot be excluded. The further increase in specific activity after dialysis also suggests the removal of other inhibitory low-molecular weight substances from the enzyme extract.

pH Profile

Initial experiments on the activity of the crude extract showed that the pH optimum in the range pH 5.0 – 8.0 was pH 7.3. The pH profile of the acetone purified extract is given in *Figure 1*. The pH optimum of this extract was found to be pH 7.2. It has been stated that the purity of the enzyme extract can affect the pH optimum (Stelzig *et al.*, 1972) but this was not evident in the case of the crude extract and a acetone purified extract from starfruit. The pH optimum of most PPO's studied have been shown to be in the range pH 5.0 – 7.0. It has also been found that pH optimum of the enzyme can be influenced by the type of phenolic substrate being oxidized. Differences in pH optima can

TABLE 1
Extraction and purification of starfruit PPO

Stage	Protein mg ml^{-1}	Specific activity U mg^{-1}	Purification fold
Crude extract	14.2	2.6 ± 1.0	1
Acetone precipitate	0.65	24.7 ± 3.1	9.5
Dialysed enzyme	0.19	56.7 ± 1.1	22

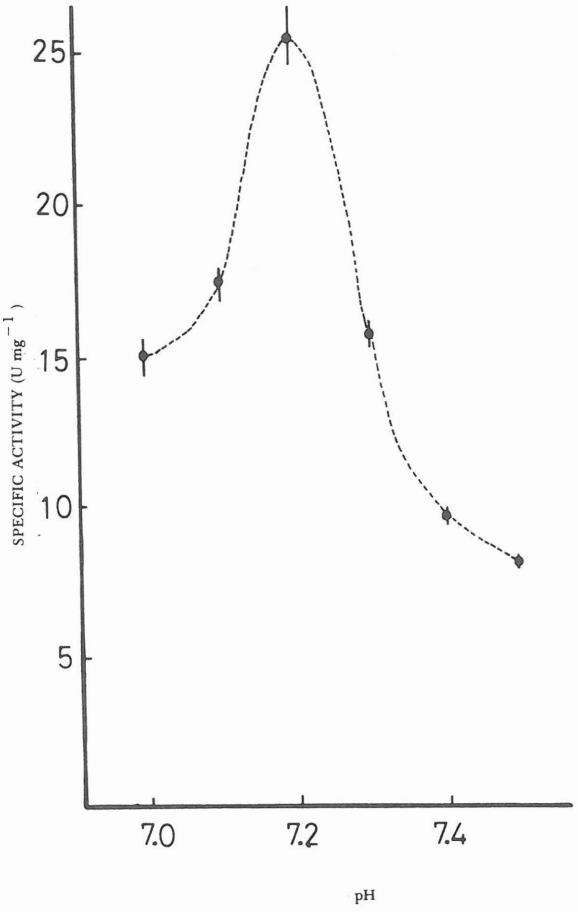

Fig. 1: pH profile of the acetone purified extract from Starfruit.

the enzyme and/or conformational changes which result in shifts in its pH optimum (Mayer and Harel, 1979).

Thermal Inactivation of Starfruit PPO

Figure 2 shows the results of the heat inactivation of the acetone purified PPO at 70°C and 75°C. As expected, the rate of enzyme inactivation increased with increasing temperature. Heat inactivation at 80°C for 2 min resulted in a 95% loss of enzyme activity.

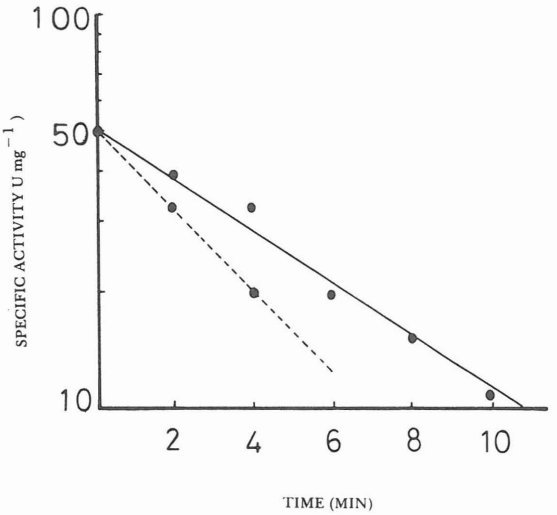

Fig. 2: Thermal Inactivation of Starfruit PPO (●——● 70°C; ●---● 75°C).

also occur when PPO is obtained from different cultivars of the fruit (Mayer and Harel, 1979).

A pH optimum of 7 has been found for a number of other PPO preparations. Among some of them with a pH optimum of 7 for PPO activity are PPO from certain varieties of apple, pear, sweet cherry, mandarin, banana, mango, mushroom and green chilly (Vamos-Vigyazo, 1981). However, it should be borne in mind that pH optimum as well as the relationship between activity and pH can differ according to genera, cultivar and substrates (Vamos-Vigyazo, 1981). Changes in pH profile of PPO activity can sometimes be accompanied by activation of the enzyme in the neutral to alkaline region of the pH curve. This is due to partial denaturation of

The linearity of the plots of log activity against time showed that heat inactivation followed first order kinetics. The half lives for heat denaturation at 70°C and 75°C were found to be 4.6 min and 3.1 min respectively. Studies into the heat inactivation of PPO from mango peel and mango (Park *et al.*, 1980; Katwa *et al.*, 1983), grape (Wissemann and Lee, 1981, Lee *et al.*, 1983), pear (Halim and Montgomery, 1978) and cherries (Benjamin and Montgomery, 1973) have shown first order kinetics of denaturation.

A comparison of the heat lability of starfruit PPO with PPO from other sources show that starfruit PPO is more susceptible to heat denaturation than PPO extracted from De

Chaunac grapes, d'Anjou pears, mango and mango peel. Half lives for heat inactivation of De Chaunac grapes were about 3.5 min and 5.4 min at 75°C and 70°C respectively (Lee *et al.*, 1983). PPO from d'Anjou pears were even more resistant to heat denaturation as shown by half lives of 6.3 min and 11.7 min at 75°C and 70°C respectively (Halim and Montgomery, 1978). Katwa *et al.* (1983) found that PPO from mango peel lost 50% of its activity by exposure to 75°C for 16 min.

The results on the heat lability of PPO are in line with the observation that PPO's are not extremely heat stable enzymes. Short exposures to temperatures of 70°C to 90°C are generally sufficient to cause irreversible inactivation of the enzyme (Vamos-Vigyazo, 1981).

K_m and V_{max} Values

The effect of varying catechol concentration is shown by the double reciprocal plot in *Figure 3*. The values of K_m and V_{max} were 0.061M and 20 U ml $^{-1}$ respectively at pH 6.8.

Luh and Phithakpol (1972) found that K_m for cling peach PPO was 0.015M while the K_m for Elberta freestone peach was 0.12M with catechol at pH 6.2 (Reyes and Luh, 1960). Mango, eggplant and banana PPO with catechol as substrate gave K_m values of 0.0082M (Katwa, 1983), 0.007M (Roudsari *et al.*, 1981) and 0.0018M (Galeazzi and Sgarbieri, 1981) respectively. These differences in K_m values suggest that the affinity of PPO towards a given substrate can vary within wide limits. The pH of the enzyme assay also affects the ultilizability of the substrate (Vamos-Vigyazo, 1981). Smaller K_m values are obtained for a stronger binding of the substrate to the enzyme. It has been suggested that differences in K_m values for PPO may be due in part to steric factors arising from differences in protein structure. A recent review by Vamos-Vigyazo (1981) lists K_m values for PPO from different fruits and cultivars which further illustrates this point.

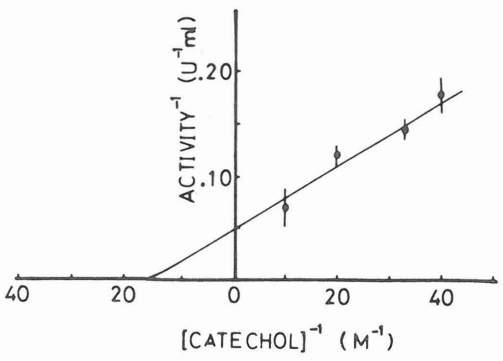

Fig. 3: Double-reciprocal plot — 1/Activity against 1/(catechol).

CONCLUSION

The paper described the characteristics of a partially purified PPO enzyme extracted from starfruit. In any work on PPO, it should be noted that there may be limitations because the characteristics can be affected by maturity of the fruit and the method of extraction and purification. Furthermore, there is a possibility of the occurrence of isoenzymes and interconversion between different forms of the enzyme during extraction and purification.

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