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Biochemical Studies on the Characters of Polyphenol Oxidase from Rambutan (*Nephelium lappaceum* L.) Peel

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Abstract: Polyphenol oxidase (PPO) from rambutan *(Nephelium lappaceum* L*)* peel was extracted and purified by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and gel filtration chromatography. The biochemical characteristics reveal that the PPO from rambutan peel has higher affinity towards catechol (K_M = 13.7mM and V_{max} = 18,105 U/ml min^{-1}) at an optimum pH of 5.9. The enzyme had an optimum temperature of 37°C and was relatively stable up to 47°C for a period of 60 minutes with almost 77% activity remaining. Among the various PPO inhibitors tested, the most effective inhibitor for the enzyme with 10mM catechol as substrate was ascorbic acid.

Key words: Polyphenol Oxidase · Rambutan · Catechol · Ascorbic Acid

INTRODUCTION

Polyphenol oxidase (E.C. 1.41.18.1) is a common copper containing enzyme responsible for melanization in animals and browning in plants also known and reported under various names based on substrate specificity [1-4]. This enzyme is widely distributed in nature and play essential roles in many vital activities [5].

In higher plants the enzyme has been localized to the thylakoid membranes of chloroplasts and other plastid organelles [6, 7]. The role of polyphenol oxidase (PPO) in plants is not yet clear, but it has been proposed that it may be involved in necrosis development around damaged leaf surfaces and in defence mechanisms against insects and plant pathogen attack [8]. It is also suggested that it may be involved in immunity reactions and in biosynthesis of plant components, also, it may plays the role as a scavenger of free radicals in photosynthesizing tissues [9, 10].

The phenomenon of enzymatic browning often occurs in fruits and vegetables and is the cause of a decrease in their sensory properties and nutritional value [11]. The browning is principally initiated by the activity of Polyphenol oxidase catalyses two distinct reactions: the *O*-hydroxylation of monophenols to *O*-diphenols which oxidized to *O*-quinones [12-14]. This enzyme is frequently reported as a latent enzyme, which can be activated *in vitro* by a number of different factors and treatments such as detergents [15-18].

Activity of PPO has been extensively reported by several authors, in apples (*Malus* sp.) [19], pears (*Pyrus* sp.) [20], peppermint (*Mentha piperita* L.) [21], coffee (*Coffea arabica* L.) [22]. However, no research has been reported on rambutan peel which it is a wast food and may be considered as a new source of natural antioxidants.

In the present study, PPO was extracted from rambutan (*Nephelium lappaceum* L.), partially purified and the characteristics of the enzyme were investigated.

MATERIALS AND METHODS

Materials: Ripe undamaged fruits were obtained from local markets in Ipoh, Malaysia. Catechol, DEAE-Sephadex A-50, Sephadex G100, caffeic acid, chlorgenic acid, phloroglucinol, Polyvinylpyrrolidine (PVP 40),

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ascorbic acid, tyrosine, thiourea, sodium metabisulphate and glutathione were obtained from Sigma-Aldrich/ USA and all other chemicals were of analytical grade.

Enzyme Extraction and Purification: Total of 300 grams of the ripe fruits peel were homogenized in 300ml of 0.1M sodium phosphate buffer (pH = 6.8)(extraction buffer) containing 15mM ascorbic acid and 0.8% polyvinilpyrrolidone and Triton X100, using T basic Ultra-Turrax (IKA Labortechnik) homogenizer in an ice bath for 3 min in 1 min intervals and extracted with the aid of magnetic stirrer for one hour. The crude extract samples were centrifuged at 50,000g for 30 min at 4°C (in refrigerated superspeed centrifuge, Sorvall RC-5C). Solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% saturation. After an hour, the precipitated proteins were separated by centrifugation at 50,000g for 30 min. The precipitate was dissolved in a small amount of 5mM phosphate buffer (pH 6.8) and dialyzed in a cellulose bag (MW cut off >12,000) at 4°C in the same buffer for 24 hours with six changes of the buffer during the dialysis analysis. In order to conduct further purification, the dialysate was transferred to a column filled with DEAE-Sephadex A-50 gel, balanced with 5mM phosphate buffer (pH 6.8). The column was eluted with the same buffer at the flow rate of 20 ml/h keeping linear gradient of NaCl concentration from 0 to 1.0M. Six milliliter fractions were collected in which the protein level and PPO activity towards catechol as substrate were monitored spectrophotometrically. The fractions which showed PPO activity were collected, concentrated and then dissolved in 6 ml of phosphate buffer (pH 6.8). The combined fractions were transferred to a glass column filled with Sephadex G100 gel. The column was then eluted with the same buffer solution.

PPO Activity Assay: PPO activity assay was determined by measuring the initial rate of quinine formation as indicated by an increase in absorbance at 420 nm [23]. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 min^{-1} [24]. The PPO activity was assayed in triplicate measurements. The sample cuvette contained 2.5 ml of 10mM catechol solution in 0.1 ml phosphate buffer (pH 6.8) and 0.05 ml of the enzyme solution. The blank sample contained only 3ml of the substrate solution.

Protein Determination: The protein content was determined in all preparations used for PPO assay by the colorimetric method described by Bradford [25]. The values were obtained by graphic interpolation calibration standard curve with bovine serum albumin (BSA) at 595 nm.

Characterization of PPO

Effect of pH on Enzyme Activity: The PPO activity as a function of pH was determined under standard conditions using various buffers in the pH buffer range 2.0-12.0 using 0.02 M catechol as the substrate. The buffer solution was prepared according to the method recommended before [26]. PPO activity was assayed as described before with catechol as the substrate. The pH value corresponding to the highest enzyme activity was taken as the optimal pH.

Kinetic Data Analysis and Substrate Specificity: The specificity of PPO extracted from rambutan peel was investigated in five different substrates like catechol, caffeic acid, chlorogenic acid, phloroglucinol and tyrosine at a concentration of 10mM. The activity of PPO extract as function of the concentration of catechol was investigated. Michaelis constant (k_M) of the PPO was determined according to Lineweaver-Burk's method.

Effect of Temperature on Enzyme Activity and Thermal Stability: PPO activity as a function of temperature was determined under standard assay conditions using temperatures from 20 to 80°C. Thermal stability of PPO was determined by heating the enzyme solution at various temperatures between 20 to 80°C for 60 minutes at pH 5.9. Residual PPO activity was measured under standard assay conditions.

Effect of Inhibitors on PPO: The inhibitory effects of ascorbic acid, thiourea, sodium metabisulphate and glutathione on PPO activity were determined. 5mM concentrations of the above compound were tested using 10mM of catechol as substrate. The corresponding control contained the same concentration of enzyme in the absence of the inhibitor.

RESULTS AND DISCUSSION

The elution profile of the PPO with DEAE Sephadex A-50 and Sephadex G100 showed that the purification fold of 2.23 relative to a protein yield of 16.98% was achieved (Table 1).

Table 1: Extraction and purification steps of PPO from rambutan (*Nephelium lappaceum* L.) pseel

Purification Steps	Protein conc. *mg/ml*	Activity *u/ml*	Specific activity *u/mg*	Purification fold	Yield (%)
Crude extract	3.66	37.88	12.41	1.00	100.00
$(NH_4)_2 SO_4$	2.28	34.76	15.47	1.24	89.70
DEAE- Sephadex A-50	1.18	19.65	17.57	1.40	50.00
Sephadex G-100	0.44	9.59	23.24	2.23	16.98

Table 2: Relative activity of PPO from rambutan (*Nephelium lappaceum* L.) peel using different substrates.

Substrate (10 mM)	Relative activity (%)
Catechol	100.00 3.110
Caffeic acid	8.87 0.820
Chlorogenic acid	3.74 0.740
Phloroglucinol	0.82 0.006
Tyrosine	0.00

The technique of gel filtration is widely used in enzyme separation. Xu *et al.,* [27] purified PPO from Henry chestnuts (*Castanea henryi)* and achieved protein recovery of 12.94%. Whereas, Selles-Marchart *et al.,* [16] purified polyphenol oxidase obtained from *Eriobotrya japonica* Lindl and achieved a protein recovery of 15%.

The activity of PPO was measured at different pH using catechol. The optimum pH of the enzyme was found to be 5.9 using catechol as the substrate.

It is seen that in general most plants show PPO activity at or near neutral pH values. The result shown in this study corresponds well with the results obtained by Urszula Gawlik-Dziki *et al*. [5] for broccoli (*Brassica oleracea* var. botrytis italic) florets (pH 5.72). It also corresponds with the results obtained by Dogan and Dogan [28] for DeChaunac apple (pH 6.0).

Though there are several compounds that are used as substrates for polyphenol oxidase, in this study we selected the most commonly used substrates such as catechol, caffeic acid chlorogenic acid phloroglucinol and tyrosine. As shown in table 2, Polyphenol oxidase showed the highest activity towards catechol (dihydroxy phenols) and the lowest activity towards phloroglucinol (trihydroxy phenol), whereas, no activity was shown towards tyrosine (monophenols). Cho and Ahn [28] used catechol in the studies of kinetic properties of PPO from potatoes, whereas, Janovitz-Klapp [29] compared the activity of PPO in apples against several substrates and obtained a similar results.

The Lineweaver-Burk plot analysis of polyphenols oxidase from rambutan peel showed that the Michaelis Menten constant (K_m) and the maximum reaction velocity (V_{max}) were 13.7mM and 18,105 U/ml min^{-1} respectively for catechol. This value for catechol was similar to that of Tea leaf (12.5 mM) [30] and also with the field bean seed (10.5 mM) [31].

The effect of temperatures between 20 and 80°C on PPO activity has shown that the optimum temperature for the PPO enzyme from rambutan peel to be 37°C. This value was similar to that obtained by Dincer *et al.,* [32] from plum (37°C). The value is also similar to that obtained by Heimdal [33] in investigations of PPO from butter lettuce (37.5).

The thermal stability profile of rambutan peel PPO, showed as residual activity after pre-incubation at the specified temperature, it was up to 47°C for a period of 60 minutes with almost 77% activity remaining. The enzyme from meddler fruits was stable for 30 minutes minuts at 60°C [34]. It has been reported that *Allium* sp. PPO was stable at 40°C for 30 min [35]. PPO from latex of *Hevea brasiliensis* was stable up to 60°C for 60 minutes [36].

The effect of various inhibitors like ascorbic acid, thiourea, sodium metabisulphate and glutathione on rambutan peel PPO with catechol as the substrate is shown in table 3.

From the K_i constants, it is concluded that the inhibition modes for thiourea, sodium metabisulphate and glutathione are non competitive and ascorbic acid is competitive. Enzymatic browning of plants and fruits may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. There are a number of inhibitors used by researchers to prevent enzymatic browning [30-32]. The inhibitory reaction mechanism differs and depends on the reducing agent that is employed.

It is seen that the compounds tested in the present study (ascorbic acid, thiourea, sodium metabisulphate and glutathione) also inhibited the action of the PPO enzyme isolated from artichoke (*Cynara scolymus* L.) heads [37].

Ascorbic acid acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial quinine by the enzyme to the original diphenol before it undergoes secondary reactions, which lead to browning [38]. Ascorbic acid has also been found to show competitive activity towards PPO isolated from peppermint [15], potato [28], thymus [39], grape [40, 41].

Two main problems were found in this investigation in the optimization of the extraction conditions of PPO from rambutan peel: the difficulty in obtaining full solubilization of the membrane-bound PPO and

Table 3: Characters of inhibition status of PPO from rambutan (*Nephelium lappaceum* L.) peel by different inhibitors

Inhibitor	K_i(M)	Type of inhibition	Inhibition (%)
Ascorbic acid	$8.2x10^{-5}$	Competitive	68
thiourea	$3.2x10^{-5}$	Non competitive	62
Sodium meta bisulphate	$3.8x10^{-5}$	Non competitive	59
Glutathione	$5.1x10^{-5}$	Non competitive	57

avoiding phenolic oxidation during and after extraction. The strength of PPO binding to membrane is variable. Therefore, in most cases, full extraction of the enzyme requires the use of a detergent such as Triton X100.

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