Isolation and Some Properties of Polyphenoloxidase from Eggplant (*Solanum melongena* Linn. Var. *melongena)* Fruit

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Abstract

Eggplant (S. melongena Linn. Var. melongena) fruit polyphenoloxidase was partially purified 17.27 fold by ammonium sulphate precipitation and gel filtration. There was a 6.8% recovery of enzyme activity following gel filtration chromatography. Catechol and pyrogallol were good substrates for the enzyme; with no activity observed with tyrosine, orcinol or resorcinol. The K_m and V_{max} values, obtained with catechol as substrate, were 11.11 mM and 29.41-units/min/mg protein respectively. Sodium azide, resorcinol, tyrosine, thiourea, and orcinol inhibited the enzyme.

Keywords: *Solanum melongena*, Polyphenoloxidase, Eggplant fruit, Catechol, Pyrogallol, Tyrosine, Resorcinol, Orcinol

Introduction

o-Diphenol: O_2-oxidoreductase (EC 1.14.18.1), polyphenol oxidase, PPO is a copper – containing enzyme that catalyses the hydroxylation of monophenols and oxidation of diphenols to o-quinones [1]. The enzyme is thought to catalyze the reactions that promote wound healing, temporarily prevent or reduce infection or reduce the rate of infection [2]. Hence, copper-deficient plants have been found to be frequently more susceptible to air-borne fungal diseases than plants with adequate copper [3]. PPO has frequently been assumed to play a role in plant defence because of its conspicuous reaction products [4]. Consequently, in many Lycopersicon and Solanum species, PPOs are highly abundant in glandular trichomes, which entrap small-bodied insects through oxidative polymerization of trichome exudates. The enzyme occurs widely in plants, and has been isolated from carrot [5]; yam [6,7,8]; sunflower seeds [9]; potato tuber [10]; broad bean leaves [11]; green olives [12]; apple [13]; cocoyam [14]; grape [15]; cocoa husk [17]; airen grape berries [18] and avocado [19]. In his review, Mayer [20] reported that the enzyme has been found in okra seeds, guava fruit and date fruit. Sharma and Ali [21] had characterized a polyphenol oxidase from *Solanum melongena*. They however failed to state which variety of the source material they had worked on. Two varieties of S. *melongena* – *melongena* and *inerme* – are prevalent in the tropics [22]. Consequently, this study is aimed at understanding some of the properties of PPO from S. *melongena* Linn. Var. *melongena*.

Materials and Methods

Materials: Eggplant, S. *melongena*, Fruits were bought from the fruit garden at Port Harcourt, Nigeria.

Chemicals: Orcinol, resorcinol, pyrogallol, tyrosine, glacial acetic acid and monobasic sodium phosphate were purchased from BDH Chemical Ltd., England, while catechol, dibasic sodium phosphate and thiourea were products of Hopkin and Williams Ltd., England, Sodium azide was purchased from Sigma Chemical Co., while Sephadex G-100 (Superfine) was purchased from Pharmacia. Sodium acetate and ammonium sulphate were purchased from Eagle Scientific Ltd., and May and Baker Nig. Ltd. respectively. All other reagents employed in this work were the best commercial grade available.

Crude enzyme preparation; 30g of eggplant fruit was cut up and blended with about 30 ml of ice-cold 0.025M sodium phosphate buffer, pH 7.0. The thick suspension was made up to 75 ml with ice-cold buffer, filtered through 2 layers of cheese-cloth and centrifuged at 10,000g for 15 mins. The dark-coloured supernatant was recovered and used as crude enzyme extract.

Ammonium sulphate precipitation: $(NH_4)_2SO_4$ crystals were added to the crude 0.025M sodium phosphate buffer (pH 7.0) extract to give 80% saturation. The mixture was vigorously shaken, and then allowed to stand for 10 minutes at ice-cold temperatures. It was then filtered through two layers of cheese-cloth, and centrifuged for 10 minutes at 10,000g. The supernatant was discarded while the pellet was dissolved in a minimal volume of 0.025M phosphate buffer, pH 7.0. Fractions of this dissolved pellet were removed for assay of enzyme activity and protein content [27]. Gel-filtration on Sephadex G-100. Sephadex G-100 was swollen in 0.025M sodium phosphate buffer, pH 7.0 for 3 days [27]. It was then packed into a column (40 x 2.5 cm) and equilibrated with the same buffer. 3 ml of the re-dissolved pellet from ammonium sulphate precipitation was applied to the column and eluted with the same buffer. Fractions of 3 ml were collected at a flow rate of 30 ml/1hr. Two adjacent fractions were pooled together and assayed for enzyme activity and protein concentration.

Protein estimation: The protein content of the enzyme xtract at different levels of purity was determined by the
rum albumin was used as standard.

Enzyme assay: Enzyme assay was determined by measuring the increase in A at 400nm with a Jenway 6100 spectrophotomeler. The reference cuvette contained 2ml of 0.025M sodium phosphate buffer, pH 7.0 and 1ml of substrate in the same buffer. The sample cuvette contained 1.9ml of 0.025M sodium phosphate buffer, 1ml of substrate and 0.1ml of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme that caused a change in A of $0.001min^{-1}$.

Substrate specificity: The following compounds at 3.3mM final concentration were tested as possible substrates of S. m*elongena* polyphenol oxidase: catechol, pyrogallol, tyrosine, resorcinol and orcinol.

Effect of inhibitors: The effect of thiourea, sodium azide, orcinol, resorcinol and tyrosine as potential inhibitors of the enzyme was determined. The modes of inhibition of the various inhibitors were determined from the Lineweaver-Burk plots of the initial velocity data. K_i values were determined from Dixon [25] plots. K_i values for uncompetitive inhibition were determined from replots of intercepts.

Effect of pH: The optimum pH for the S. *melongena* PPO was determined by measuring the enzyme activity at different pH values (4.0-8.0) using 3.3 mM catechol as substrate. 0.025M sodium acetate buffer was used for the pH range 4.0-5.6, while 0.025M sodium phosphate buffer was used for the pH range 5.7-8.0.

Optimum temperature: The optimum temperature for the activity of the enzyme was determined by incubating reaction mixtures, each containing 3.3 mM catechol at different temperatures for 5 mins. 0.1 ml portions of enzyme were then added to initiate each reaction. Enzyme activity was measured as described above.

Thermal inactivation: Various samples of the enzyme were incubated at different temperatures in a water bath. Aliquots were removed at various time intervals for each temperature, and assayed for PPO activity.

Results and Discussion

Enzyme isolation: Table 1 shows the various steps used for the partial purification of polyphenol oxidase from eggplant fruit. At the end of the $(NH_4)_2SO_4$ precipitation step, 2.33 fold purification and 79.8% recovery of total enzyme activity were achieved. Following this step by gel – filtration on sephadex G-100 gave a final purification of 17.27 fold and 6.8% recovery of total enzyme activity (table 1). Ben-Shalom et al. [12] achieved a similar purification of 23.3 fold with 12% recovery on the employment of gel filtration chromatography for the resolution of PPO from green olives. Similarly, on the employment of gel filtration to PPO from D. *bulbifera,* there was 10% recovery of the enzyme, with a purification of 22 fold [6].

Substrate specificity: This enzyme was found to use catechol and pyrogallol as substrates. On the other hand, tyrosine, resorcinol and orcinol could not be oxidized by the enzyme within one minute reaction time. This is similar to the finding for the *Xanthosoma sagittifolium* enzyme [14] except that catechol was the best of those substrates tested for the S. *melongena* enzyme (table 2) while pyrogallol was the best for the *Xanthosoma* enzyme. The enzyme under study oxidized catechol with a V_{max} of 29.41 units/min/mg protein. Furthermore, the apparent K_m of 11.11mM found in this work for the S. *melongena* PPO with catechol as substrate (Fig.1) compares favourably with the 10mM reported for litchi fruits pericap enzyme with 4-Methyl catechol as substrate [23]; 10.25mM found for the enzyme from *Dioscorea rotundata* [24]; 13 mM and 9mM found for the enzymes from D. *rotundata* and D. *bulbifera* respectively [8], each with catechol as substrate. The inability of the enzyme to oxidize tyrosine suggests that it lacks cresolase activity.

Effect of temperature: An optimum temperature of 40^0C found in this work for S. *melongena* PPO (Fig.2) corresponds to the same value found for the enzyme from D. *rotundata* [24] and D. *bulbifera* [6]. Similar values of 45^0C and 37^0C were found for the enzyme from sunflower seeds [9] and cocoyam [14] respectively. After 40 minutes incubation at 30^0C, 40^0C, 50^0C; the S. *melongena* PPO retained 88%, 70% 59%, and 30% of its original activity, respectively (Fig 2). It however, retained only 10% of its original activity after incubation at 70^0C and 80^0C for 20 minutes and 15 minutes respectively. This enzyme is therefore less heat labile than the D. *rotundata* enzyme, which lost all its activity after 5 minutes incubation time at 80^0C [24]; the D.*bulbifera* enzyme which lost all its activity within the first 5 minutes of incubation at 70^0C [6]; the cocoyam enzyme which lost all its activity after 20 minutes incubation time at 68^0C [14]. It is however, more heat labile than the enzyme from green olives, which still retained about 50% of its original activity after 19 minutes incubation time at 75^0C [12].

Effect of pH: The pH profile of S. *melongena* PPO with catechol as substrate showed a pH optimum of 7.0. This same value was found for the yam enzyme [6, 8 and 24]; the litchi fruits pericarp enzyme [23], and the oil bean seed enzyme [27]. It is also similar to 7.4 found for the cocoyam enzyme [14]. The pH optimum of 4.5 found for the enzyme from green olives [12] however, differs significantly. Sharma and Ali [21] had found two pH maxima at 6.5 and 7.5 with catechol as substrate for PPO from S. *melongena.* They, however, did not state the variety of the source material used.

Effect of inhibitors: Table 3 shows the mode of inhibition by some compounds of the reaction catalysed by S. *melongena* PPO with catechol as substrate. Thiourea inhibited the enzyme

Table 1: Purification Profile of Eggplant PPO

Treatment	Volume (cm^3)	Protein conc. (mg/cm^3)	Total Protein (mg)	Total activity (units x 10^3)	Specific activity (units x 10^3/mg protein)	Yield (%)	Purification fold
Crude 0.025M Phosphate buffer extract	250	12.8	3200	22250	6.96	100.0	1.00
80%$(NH)_2 SO_4$ precipitate	40	27.4	1096	17760	16.20	79.8	2.33
Gel-filtration through sephadex G-100	84	0.15	12.6	1512	120.00	6.8	17.27

Table 2: Substrate Specificity of Eggplant Fruits PPO

Substrate*	Max λ of Oxidation	λ used	V(ΔA_{400}/min/mg)	Relative Activity
Catechol	400	400	6.797	100
Pyrogallol	334	364	4.219	62.07
Tyrosine	472	472	0	0
Resorcinol	-	400	0	0
Orcinol	-	400	0	0

**Concentration of each substrate used = 3.3 mM.*

Table 3: Some established inhibitors of eggplant fruit PPO

Inhibitors	Mode of inhibition	Inhibition constant, k_i (mM)
Sodium azide	Non-competitive	6.0
Resorcinol	Competitive	2.2
Tyrosine	Competitive	3.8
Thiourea	Uncompetitive	0.75
Orcinol	uncompetitive	2.40

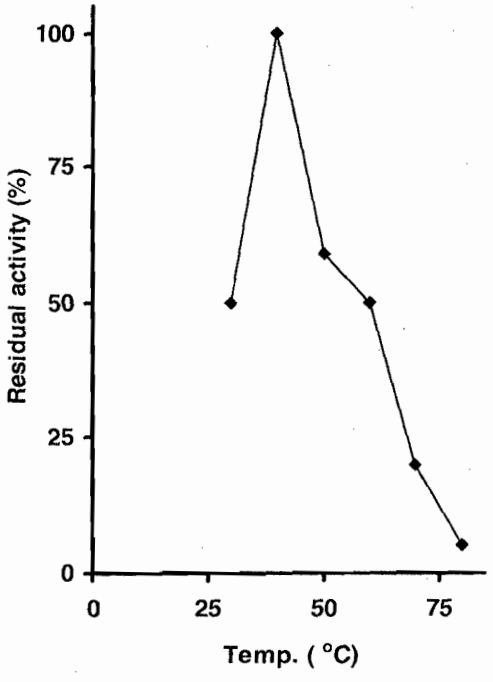

Fig. 1 : Variation of the activity of eggplant fruit PPO with temperature. Observed optimum temperature = 40 °C

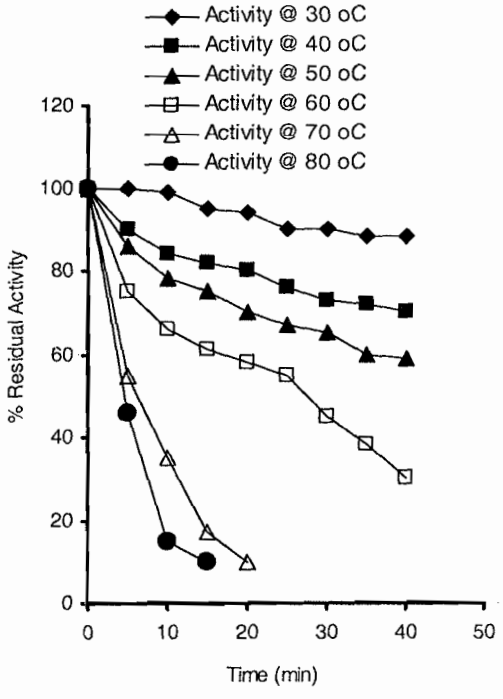

Fig. 2: Time curves for heat inactivation of eggplant fruit polyphenol oxidase. enzyme samples were incubated for periods indicated and residual activity determined

uncompetitively with respect to catechol. This same pattern of inhibition by thiourea was found for the enzyme from cocoyam [24]. Sodium azide which inhibited the binding of catechol to S. *melongena* PPO non-competitively, also gave a similar pattern of inhibition for the oil bean seed enzyme [27] and D. *bulbifera* enzyme [7]. Furthermore, tyrosine competitively inhibited the binding of catechol to the S. *melongena* enzyme. A similar result was found by Chilaka *et al* [27] for the oil bean seed enzyme. Similarly, resorcinol which competitively inhibited the binding of catechol to the S. *melongena* enzyme was found to give a similar pattern of inhibition with the D. *bulbifera* enzyme [7] and the oil bean seed enzyme [27]. Orcinol however, uncompetitively inhibited the binding of catechol to the S. *melongena* enzyme, and gave the same pattern of inhibition with the oil bean seed enzyme [27].

It however, gave a mixed pattern of inhibition with the D. *bulbifera* enzyme [7]. Chilaka *et al* [27] had suggested that it is possible that inhibition by tyrosine, resorcinol and orcinol of PPO is due to their different o-quinones, and since the structures are different, especially orcinol differing from resorcinol by possession of a methyl group, the mode of inhibition may be different.

In summary, the enzyme under study has been found to resemble polyphenol oxidase of other higher plants that have been reported on; particularly with regards to its pH optimum, substrate specificity and effects of inhibitors.

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