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PURIFICATION AND SOME PROPERTIES OF POLYPHENOL OXIDASE FROM THE YAM TUBERS, *DIOSCOREA BULBIFERA*

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Key Word Index—*Dioscorea bulbifera*; Dioscoreaceae; yam tuber; polyphenol oxidase.

Abstract—In a comparison of the polyphenol oxidase activity of various species of yam tubers the greatest enzyme activity was found in *D. bulbifera*. The enzyme was purified from acetone powder extracts of this plant. Ammonium sulphate fractionation, followed by ion exchange chromatography and gel filtration gave 22-fold purification. The final product gave a single band on polyacrylamide disc gel electrophoresis. The purified enzyme showed activity towards catechol, pyrogallol and DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) and had a MW 115000 ± 2000. It was characterized by response to various inhibitors. β-Mercaptoethanol, dithioerythritol, L-cysteine, sodium metabisulphite and KCN inhibited strongly.

INTRODUCTION

Browning reactions which occur after infliction of a mechanical injury to some plant tissues like fruits, tubers and vegetables are initiated by the enzyme, polyphenol oxidase (*o*-diphenol: O_2 oxido-reductase, EC 1.10.3.1). The enzyme has been purified and studied from peaches [1], egg plants and avocado [2], carrots [3], apples [4], potato [5] and banana [6]. Yam tubers constitute an important source of carbohydrate in the diet of people in most parts of west Africa. Rapid darkening occurs when the tissues are cut, peeled or crushed during processing for food or for storage. Kahn [7] found that there was a correlation between the amount of polyphenol oxidase in three varieties of avocado with the degree of browning observed in the freshly cut fruit. Recent progress in the study of plant polyphenol oxidases has been reviewed in ref. [8]. This study is aimed at the isolation and study of the polyphenol oxidase from a species of yams that is known to show the most intense browning phenomenon on cutting or peeling. Knowledge of the potent inhibitors of polyphenol oxidase from this source might lead to finding ways of processing yam powders that would retain the original whitish colour of the intact tuber. This would be of economic importance to the food storage industry of Nigeria.

RESULTS AND DISCUSSION

Levels of polyphenol oxidase activity in various types of yam tubers

The data in Table 1 show various levels of enzyme activity in crude extracts of the six types of yam commonly cultivated in the eastern States of Nigeria. *D. bulbifera* contains by far the highest levels of polyphenol oxidase activity. It is no wonder that this species of yam turns brown a few seconds after it has been cut open or peeled. Because of this very high level of polyphenol oxidase activity detected in this type of yam, it was selected as a source for the subsequent isolation and purification of this enzyme.

Table 1. Levels of polyphenol oxidase in various yam (*Dioscorea*) tubers

Type of yam	Enzyme activity (10^3 units/mg)
D. bulbifera	43.9
D. caynensis	9.8
D. rotundata	3.0
D. esculenta	0.9
D. dumentorum	0.3

Enzyme purification

Table 2 shows the purification profile of polyphenol oxidase from *D. bulbifera* with catechol as substrate. As reported in ref. [13] the acetone powder could be stored for long periods at −20° with little loss of activity.

Ion exchange chromatography and gel filtration

Unlike the catechol oxidase from green olives [9], the polyphenol oxidase of Royal Ann cherries [10], banana [11], mushroom [12] and grape [13], after ion exchange chromatography and gel filtration of the partially purified enzyme gave a single peak of activity towards catechol as substrate. The concentrated pooled fractions showing enzyme activity after ion exchange chromatography were rechromatographed on Sephadex G-100. One large protein peak (fraction 26) and a shoulder near the void volume of the column (fractions 20–24) were obtained. Fractions 20–24 were pooled and concentrated by vacuum dialysis. With the process of purification employed, there was 10% recovery of enzyme activity with a purification of 22-fold. Ben-Shalom *et al.* [9] have achieved purification of 239-fold with 7.5% recovery in their preparation of catechol oxidase from green olives, while Benjamin and Montgomery [10] recovered 7% yield of polyphenol oxidase from Royal Ann cherries with 63-fold purification. Comparing the sp. act. of the buffer extract of the acetone powder in Table 2 with that of crude buffer enzyme extract in Table 1, there is no doubt that a lot of enzyme was lost

Table 2. Purification of polyphenol oxidase from *D. bulbifera*

Purification step	Total protein	Sp. act. ($\times 10^3$ units/mg)	Yield (%)	Purification (fold)
1. Buffer extract of acetone powder	1860	0.40	100	1
2. Solution of acetone ppt	696	0.72	66.8	1.8
3. Dialysed solution of 80% $(NH_4)_2SO_4$ ppt	217	1.86	54	4.7
4. DEAE-cellulose (peak fractions)	49	2.61	16.9	6.5
5. Sephadex G-100 (peak fractions)	8.6	8.8	10	22

either in the process of preparation of the acetone powder or during buffer extraction of this powder. However, the initial preparation of the acetone powder of this plant material helps to eliminate most of the coloured materials of the tissue that would otherwise have interfered with the assay for enzyme activity.

Disc gel electrophoresis (PAGE)

The product from gel filtration on a Sephadex G-100 column gave a single sharp band on staining for protein after PAGE. Two of the gels that were stained for enzyme activity gave single light pink bands in positions corresponding to the positions of the protein bands which were detected on the other gels. This preparation of polyphenol oxidase appears to be homogeneous.

Substrate specificity

The enzyme preparation oxidized catechol, DOPA, and pyrogallol but not *p*-cresol and L-tyrosine. The assay was as in ref. [10]. Table 3 shows the relative activities of the pure enzyme with these compounds as substrate. Catechol was by far the best substrate. The Royal Ann cherry polyphenol oxidase is similar to this enzyme in its substrate specificity. However, pyrogallol was the best of the substrates tested for this enzyme. As with the *D. bulbifera* enzyme, it could not oxidize tyrosine and *p*-cresol [10]. The apparent K_m and V_{max} values are indicated in Table 3. The K_m value of 9.1 mM found for catechol is similar to the 8.3 mM value reported in ref. [9] for the catechol oxidase from green olives. However, DOPA had a lower K_m value of 4.8 mM compared with the 10 mM for the green olive enzyme. For the polyphenol oxidase from cling peaches, [14], a K_m value of 15 mM for catechol has been reported.

Effect of inhibitors

Table 4 shows the effects of various inhibitors on the enzyme activity with catechol, pyrogallol and DOPA as substrates. With catechol as substrate, each of the thiol reagents such as β-mercaptoethanol, dithioerythritol, L-cysteine and sodium metabisulphite, at the concentrations indicated, showed varying time-lags before the manifestation of some enzyme activities. This was not the case with the other two substrates. However, with DOPA as substrate and these same compounds as inhibitors, there tended to be a marked recovery of activity about 90 sec after the start of the reactions. Benjamin and Montgomery [10] had reported that several compounds e.g. sodium diethylthiocarbamate (DETC), dithiothreitol, potassium metabisulphite, known to reduce enzymic browning of fruits delayed the start of reaction catalysed by Royal Ann cherry polyphenol oxidase from 2 to 5 min. β-Mercaptoethanol, dithioerythritol, sodium metabisulphite, L-cysteine and KCN appear to inhibit the *D. bulbifera* enzyme at very low concentrations. The effect of the chelating agent, EDTA, on the enzyme activity was minimal. This is similar to the finding of ref. [14] for the cling peach enzyme. Knapp [15] had also reported that EDTA had no measurable effect on polyphenol oxidase activity in egg plant even at high concentration. Luh and Bulan [14] had speculated that the pH of the reacting mixture may affect the affinity of EDTA towards copper which is an important part of polyphenol oxidase. It would mean that under the conditions of our experiment (pH 7.0) the chelation is certainly slight. Reyes and Luh [16] reported that DETC another copper chelating agent, inhibited both polyphenol oxidase and peroxidase activity of freestone peaches, though peroxidase is an iron-containing enzyme. This was further

Table 3. Substrate specificity of *D. bulbifera* polyphenol oxidase and kinetic constants (K_m and V_{max} values were determined from Lineweaver–Burk plots of initial velocity data)

Substrate	Assay wavelength (nm)	Activity ($\times 10^3$ units/mg protein)	K_m (mM)	V_{max} ($\times 10^3$ units/min/per mg)
Catechol	395	8.8	9.1	28.6
Pyrogallol	334	3.5	6.3	9.1
DL-DOPA	460	2.2	4.8	3.6
L-Tyrosine	472	—	—	—
p-Cresol	395	—	—	—

Table 4. Inhibitors of *D. bulbifera* polyphenol oxidase

Inhibitor	Catechol as substrate (3.3 mM) (% inhibition)	Pyrogallol as substrate (3.3 mM) (% inhibition)	DOPA as substrate (6.6 mM) (% inhibition)
0.05 mM β-Mercaptoethanol	41.7	35.0	85.0
0.05 mM Dithioerythritol	81.6	95.0	61.5
1.0 mM EDTA	7.8	0.0	0.0
0.5 mM Thiourea	85.4	5.0	11.5
0.1 mM Sodium metabisulphite	34.0	55.0	38.5
0.2 mM KCN	70.0	62.5	50.0
1.0 mM NaN_3	30.0	7.5	15.4
0.5 mM L-Cysteine	61.0	22.5	7.7
1.0 mM *p*-Cresol	42.0	32.5	42.3
1.0 mM L-Phenylalanine	21.0	12.5	11.5

Enzyme 0.1 ml containing 11.6 μg of protein was pre-incubated with a mixture of the inhibitor and 0.25 M KPi buffer pH 7 for *ca* 2 min at 25°. Reaction was started by addition of the appropriate concentration of the substrate.

supported by the finding in ref. [17]. The lag period observed in the reaction when this compound was used as inhibitor has been explained in ref. [14] as possibly due to the product of the enzymic oxidation being temporarily reduced by the inhibitor. No DETC was available for the study on the *D. bulbifera* enzyme but it is likely that the lag periods noticed in the reactions in the presence of the thiol-containing compounds can be accounted for on the basis of this explanation.

Effect of pH

A plot of *D. bulbifera* polyphenol oxidase activity against pH gave a bell-shaped curve with a maximum at pH 7.0. This is similar to that of one of the polyphenol oxidases of the Royal Ann cherry [10]. pH optima near neutral have been found for the enzymes from various sources: egg plant [18], cranberry [19], bean leaf [20] and peach [1]. On the other hand, the apple enzyme has been found to have two pH optima, 5.2 and 7.3, with the activity at pH 5.2 several times greater than that at pH 7.3 [21]. Luh and Bulan [14] reported a pH optimum of 6 for polyphenol oxidase from Halford peaches, while Balasingam and Ferdinand [22] obtained pH 5 as the optimum for the enzyme from potatoes, and pH 4.5 is reported for the green olive enzyme in ref. [9]. The indication from this is that the pH optimum of polyphenol oxidase varies depending on the source from which it is prepared.

MW

The MW of the purified *D. bulbifera* enzyme was determined to be 115000 ± 2000 by gel filtration according to the method of Andrews [23]. The column of Sephadex G-100 (2.5 × 75 cm) was first calibrated with the following proteins: cytochrome *c* (13000); bovine serum albumin (65000), acid phosphotase (85000) and LDH (150000). A MW of 42000 for the green olive catechol oxidase has been reported [9] while a value of 36000 has been indicated for *o*-diphenol oxidase from potatoes [22]. Robb *et al.* [24] have also reported a MW 33000 ± 2000 for broad bean tyrosinase, while Bull and Carter [25], working on tyrosinase from *Aspergillus nidulans*, indicated a monomer MW of 130000 in equilibrium with the tetramer of MW 5.2×10^5. Padron *et al.* [26] also reported the presence of multiple forms of polyphenol oxidase from *Musa cavendishii* on gel filtration on Sephadex G-100. The two main peaks corresponded to MWs of *ca* 60000 and 12000. MWs of from 14000 to 126000 have been reported for various aggregates of the avocado enzyme [27] while the tea leaf enzyme has a MW of 144000 ± 16000 [28], close to that of the *D. bulbifera* enzyme. The polyphenol oxidases are groups of different enzymes with different substrate specificities. It is not surprising therefore that the MWs would vary, depending on the source and method of preparation.

Effect of temperature

The optimum reaction temperature for the *D. bulbifera* polyphenol oxidase was found to be 40°. At 40° there was no measurable loss in enzyme activity even after 30 min incubation. At 50° the loss in activity appeared to follow a first-order process for up to 30 min while at 55° this was the case for the first 20 min (50% loss in 18 min). At the higher temperatures of 60° (50% loss in 10 min) and 65° (50% loss in 3 min) the heat denaturation did not follow a first-order process at all. At 70°, all activity was lost within the first 5 min of incubation. The work of Benjamin and Montgomery [10] on Royal Ann cherry polyphenol oxidase showed that both isoenzymes still retained 20% activity after incubation at 75°. The *D. bulbifera* enzyme is thus somewhat more heat labile than these cherry enzymes.

EXPERIMENTAL

Materials. The yams, *D. bulbifera*, were purchased from Ibagwa market at Nsukka.

Chemicals. Succinic acid, catechol, β-mercaptoethanol and L-cysteine were from BDH chemicals. NaOH, Me_2CO, K_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$ were from Merck. Tyrosine, acid phosphatase, LDH, and cytochrome *c* were from Sigma. Sephadex G-100 was obtained from Pharmacia. All other chemicals were Analar grade.

Crude enzyme prepns from various tubers. Crude enzymes from six classes of yams were prepared according to the method described in ref. [29].

Enzyme purification

Me_2CO *powder. D. bulbifera* (200 g) was cut into small pieces and soaked in 500 ml of Me_2CO and the mixture left 18 hr at −15°. It was then macerated and filtered under vacuum at room temp. The residue was once more blended with 500 ml of cold Me_2CO and filtered. The residue, the Me_2CO powder was spread out on clean sheets of paper and left to dry in air.

Buffer extraction. 50 g of the Me_2CO powder plus 0.2 g SDS were suspended in 400 ml 0.025 M KPi buffer, pH 7, and blended. The thick suspension was squeezed through 3 layers of cheesecloth and centrifuged at 20000 *g* for 15 min. The dark-straw-coloured supernatant contained enzyme activity.

Me_2CO *precipitation.* To 300 ml of the buffer extract were added an equal vol. of cold Me_2CO. The mixture was stirred for 15 min and spun at 20000 *g* for 10 min at 4°. The slimy ppt. was dissolved in 0.025 M KPi buffer, pH 7.

$(NH_4)_2SO_4$ *precipitation.* Solid $(NH_4)_2SO_4$ was added to the soln from the above step to 80% satn and the mixture stirred for 30 min at 4°. The resulting ppt was isolated by centrifugation at 20000 *g* for 10 min at 4°. It was dissolved in a minimal vol. of 5 mM Tris–HCl buffer, pH 3.8,. It then was dialysed for 18 hragainst 3 l. of the same buffer with three changes.

Ion exchange chromatography. The dialysed extract was applied to a DEAE-cellulose DE 52 column (2.5 × 38 cm) pre-equilibrated with the same buffer. It was washed with *ca* 180 ml of the same buffer and eluted with a linear gradient of 300 ml of 5 mM Tris–HCl buffer, pH 8.3. It then was dialysed for 18 hr against 3 l. of the same buffer with three changes.
polyphenol oxidase activity.

Gel filtration. The fractions which showed enzyme activity after ion exchange chromatography were concd to 6 ml by vacuum dialysis against 0.025 M KPi buffer, pH 7 and applied to a column of Sephadex G-100 (2.5 × 75 cm) previously equilibrated and subsequently eluted with the same buffer. Fractions (6 ml) were collected and assayed for enzyme activity. Those that showed activity were pooled, concd by vacuum dialysis, and used as purified enzyme for subsequent studies.

Assay of enzyme activity. Enzyme activity was determined according to the method of ref. [10]. One unit of enzyme activity was defined as the amount of enzyme that caused a change in *A* of 0.001/min. The increase in *A* was linear with time for only *ca* 90 sec.

Protein determination. The protein contents of enzyme prepns were determined by the method of ref. [30] with bovine serum albumin as standard.

PAGE. This was performed essentially as described in ref. [31] using tubes of 0.5 cm i.d. filled to 7 cm with 7.5% polyacrylamide in 0.05 M Tris–glycine buffer, pH 8.3. The electrode buffer was 5 mM Tris–glycine buffer, pH 8.3. The gels were run at 5 mA/tube for 2.5 hr. After the run, two of the gels containing the enzyme prepn were stained for protein with 0.001% (w/v) bromophenol blue; while another two were stained for polyphenol oxidase activity by addition of 3.8 ml 0.25 M KPi buffer, pH 7 plus 2 ml 10 mM catechol.

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