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PURIFICATION AND SOME PROPERTIES OF *o*-DIPHENOLASE FROM WHITE YAM TUBERS

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Key Word Index—*Dioscorea rotundata*; Dioscoreaceae; white yam tuber; *o*-diphenolase.

Abstract—In a screening of 10 cultivars of some popular food yam species for *o*-diphenolase activity, highest enzyme activity was found in *Dioscorea bulbifera* (cv Adu) followed by *D. rotundata*, *D. cayenensis* and *D. alata*. *o*-Diphenolase from *D. rotundata* (cv Zaria) was purified 122-fold from acetone powder preparations, using ammonium sulphate fractionation, ion exchange chromatography and gel filtration. On polyacrylamide disc gel electrophoresis, the purified enzyme gave a single sharp protein band which also coincided with the enzyme activity band. The purified enzyme and its crude forms from the other cultivars showed activity towards catechol, chlorogenic acid, DOPA and pyrogallol. Significant cresolase activity was essentially absent. The enzyme was inhibited strongly by dithiothreitol, diethyldithiocarbamate, iodosobenzoate, L-ascorbate, potassium cyanide, 2-mercaptoethanol and L-cysteine. Inhibition by the last three was competitive while that by sodium azide was uncompetitive. pH-activity studies identified two groups with pK_a of 4.7 and 7.2 involved in enzyme catalysis.

INTRODUCTION

In many edible plant products including the white yam (*Dioscorea rotundata*) tubers, the formation of several shades of colours generally termed browning observed to occur during handling, packaging and processing, is attributed to the activity of the enzyme, *o*-diphenolase (*o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) on its phenolic substrates [1]. Although this enzyme has been purified and studied from tobacco leaves [2], banana [3], potato [4], peaches [5], cherries [6], mangoes [7], apples [8] and carrots [9], there is an obvious lack of information of the yam tuber enzyme. Indirect reports of the association of *o*-diphenolase activity with browning in yams have appeared [10, 11]. Recently Adamson and Abigor [12] reported unusual transformations involving crude *o*-diphenolase of the water yam (*Dioscorea alata*) during postharvest storage of the latter. Anosike and Ayaebene [13] have very recently succeeded in purifying polyphenol oxidase from *D. bulbifera*. The current status of research on plant *o*-diphenolases has appeared in a comprehensive review [14]. Yams are second only to cassava in importance as a source of calories for millions of West African peoples [15] and the white yam is easily the most popular food yam grown in much of West Africa. In Nigeria, 25–40% of annual yam harvests are usually lost during storage as a result of serious pest attacks and unfavourable storage environments [16]. Consequently, yam processing as a means of extending the storage life of raw food yams is beginning to appeal to some food processing industries. The preference of Nigerian consumers for white processed yam products and the disastrous experience of Food Specialty (Nig.) Ltd. in the trial production and marketing of 'poundo yam' a few years back, underscore the need for a systematic investigation of the postharvest biochemistry of the yam tuber, with particular reference to the browning principles, before it can successfully be used as raw material in food processing. This study was therefore aimed at the purification and study of the browning enzyme, *o*-diphenolase, from the most important and abundant species of food yam grown in West Africa. A sound knowledge of the behaviour of the *o*-diphenolase–diphenol system of the white yam under various conditions will be of use to the Nigerian food processing industry.

RESULTS AND DISCUSSION

Levels of o-diphenolase in various cultivars of some yam species

Table 1 shows the levels of *o*-diphenolase in 10 cultivars from four yam species grown for food in different parts of Nigeria. The data show that *D. bulbifera* (cv Adu) has the highest while the two *D. alata* cultivars studied have the lowest *o*-diphenolase activity. Anosike and Ayaebene [13] also found that of the five food yams species they examined, *D. bulbifera* contained the highest level of *o*-diphenolase activity. Their enzyme activity figure of 43.9×10^3 units/g is similar to ours in this work. Our experience in using these yam cultivars indicates that the rapidity and intensity of browning in bruised or

Table 1. Levels of crude *o*-diphenolase from some yam cultivars grown in Nigeria

Yam species/cultivar	Enzyme activity × 10^3 (units/mg)
Dioscorea rotundata	
cv Zaria (purified enzyme)	22.1
Amala	26.9
Orhohozar	9.1
Omi	31.6
Obiaturugo	37.6
D. alata	
Igiehua	4.95
Mbala	4.8
D. cayenensis	
Oku	19.2
Ikpen	8.1
D. bulbifera	
Adu	41.1

cut tubers correlate very closely with the levels of *o*-diphenolase observed in them. *o*-Diphenolase activity and total polyphenolic content should be considered in selecting ware yams for processing. Our choice of *D. rotundata* as a source of *o*-diphenolase in this work was based on the dietary popularity of cultivars of this yam species and their preference as raw materials for various processed yam products.

Enzyme purification

Table 2 shows the purification profile of *D. rotundata* (cv Zaria) *o*-diphenolase using catechol to monitor enzyme activity through the various purification steps. The use of acetone powder reduced browning which tended to diminish the activity and interfere with the assay of crude enzyme extracts. When gel filtration was the first chromatographic step, the viscous and slimy nature of the crude ammonium sulphate precipitated enzyme extract interfered with the entry of the protein mixture into the column. Moreover, there appeared to be an obvious degradation of the Sephadex G-200 material. As a result, the chromatographic steps were carried out as shown in Table 2 to give a 122-fold purification. This is considerably greater than the purification of 22-fold obtained for the *D. bulbifera* enzyme by Anosike and Ayaebene [13]. All three chromatographic steps resulted in a single peak of enzyme activity towards catechol as substrate, unlike the reported experience with the enzyme from grapes [17], banana [18], green olives [19], mushroom [20] and Royal Ann cherries [21]. The pooled active peaks in the three chromatographic steps were fractions 11–19, 23–25 and 19–22, respectively. Early attempts to use calcium phosphate gel-cellulose prepared according to Koike and Hamada [22] to purify this enzyme did not result in significant purification and was therefore abandoned.

Polyacrylamide disc gel electrophoresis (PAGE)

PAGE of the enzyme from the second ion exchange step gave a single sharp protein band upon staining the gels with Coomassie blue. Staining some of the gels for *o*-diphenolase activity resulted in a perfect match between the protein and activity bands on the gels. The *o*-diphenolase preparation therefore appeared to be sufficiently pure for subsequent studies. Anosike and Ayaebene reported similar results for the *D. bulbifera* enzyme.

Substrate specificities

The purified *D. rotundata* *o*-diphenolase showed activity on catechol, chlorogenic acid, DOPA and pyrogallol with the intensity of activity decreasing in that order. *p*-Cresol, 3,4,5-trihydroxybenzoic acid, L-tyrosine, quinol and *p*-coumaric acid were not oxidized at all. While the purified *o*-diphenolase possessed no cresolase activity, the crude enzyme preparations from the cultivars listed in Table 1 exhibited some, though insignificant, cresolase activity with the Orhohozar cultivar of *D. rotundata* possessing the highest cresolase activity. It appears that the level of this activity is generally low in yams and is easily lost during purification of *o*-diphenolase

Table 2. Purification profile of *D. rotundata* *o*-diphenolase

Purification step	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude *D. rotundata* extract from Me_2CO powder	3290	81	100	1
20–85% $(NH_4)_2SO_4$ precipitation	2530	105	99	1.3
Ion exchange chromatography on DEAE–Sephadex A-50	919	104	48	1.72
Gel filtration on Sephadex G-20	15	8060	46	100
Second ion exchange chromatography on DEAE–Sephadex A-50	2.42	9900	9.1	122

activity. Catechol was still found to be the best substrate for all these enzyme preparations. The purified enzyme from *D. bulbifera* [13] and Royal Ann cherries [21] behaved in a similar fashion toward these substrates, although for the Royal Ann cherry *o*-diphenolase, pyrogallol proved to be the best substrate. The apparent K_m values of the purified and crude *o*-diphenolase preparations with catechol as substrate range from 8 mM for *D. bulbifera* enzyme to 17 mM for *D. alata* (cv Igiehua). The purified *D. rotundata* enzyme has a K_m of 13 mM which differs substantially from the value of 9 mM recently reported for purified *D. bulbifera* enzyme by Anosike and Ayaebene [13]. It would appear from the results of K_m studies that the magnitude of the K_m values obtained were not in any way related to either the species or cultivars of yam used as source of the enzyme. Apparent K_m values for catechol of 8, 15, 14, 29 and 0.7 mM have been reported for the enzyme from green olives [19], cling peaches [23], cocoa husk [24], tea leaves [25] and *Solanum melongena* [26], respectively.

Effect of inhibitors

Table 3 shows the effects of various inhibitors on *o*-diphenolase activity with catechol as substrate. The thiol reagents, L-cysteine, sodium metabisulphite, dithiothreitol and 2-mercaptoethanol and diethyldithiocarbamate (DEDTC) and L-ascorbate, all at 1 mM concentration inhibited the enzyme completely. DEDTC at very low concentrations has been reported to inhibit the *o*-diphenolase from broad beans [27], peaches [5], Royal Ann cherry [6] and mangoes [7] presumably by complexing the copper prosthetic group of the enzyme. Sodium metabisulphite was found to be the most effective inhibitor of mango *o*-diphenolase while ascorbate was the least potent [7]. The inhibition by thiol compounds may be due to an addition reaction with the quinones to form thioethers, combination with copper required for enzymatic activity [26], or general action as reducing agents [1, 28]. Ascorbate which acts more as an antioxidant than as an enzyme inhibitor appears to inhibit by reducing the initial quinone formed by the enzyme reaction before this quinone undergoes secondary reactions leading to browning [2, 29]. EDTA showed practically no inhibition under the assay conditions used. Sharma and Ali [26] made a similar observation with *o*-diphenolase from *Solanum melongena*. It is probable that the copper prosthetic group is buried within the three-dimensional structure of the enzyme protein, or that the stability of the complex of copper ion with the enzyme protein is much stronger than that of copper with EDTA at the assay pH of 6.8. For most of the inhibitors tried, inhibition was generally greater with purified than crude enzyme. Lineweaver–Burk analysis of inhibition data revealed that the inhibition by 2-mercaptoethanol, L-cysteine and potassium cyanide was competitive with K_i of 0.5, 0.1 and 0.2 mM, respectively, while that by sodium azide was uncompetitive with a K_i of 1.8 mM.

Molecular weight

The MW of purified *D. rotundata* *o*-diphenolase was determined to be 107000 ± 5400 by gel filtration chromatography on a Sephadex G-200 column (2.6 × 40 cm) which was first calibrated with the proteins: catalase (225000), haemoglobin (65000), D-(−)-lactate dehydrogenase (146000), bovine serum albumin (67000), egg albumen (54000) and cytochrome *c* (12500). Anosike and Ayaebene [13] recently obtained a MW of 115000 ± 2000 for *D. bulbifera* polyphenol oxidase by gel filtration on Sephadex G-100. The MW's of the predominant forms of mushroom tryrosinase have been reported to lie between

Table 3. Inhibition of *D. rotundata* *o*-diphenolase

Inhibitor (1 mM)	Inhibition (%)	
	Crude enzyme	Purified enzyme
N-Acetylimidazole	5.6	8.1
Dithiothreitol	100	100
Diethyldithiocarbamate	100	100
Iodoacetic acid	10	16.2
Iodosobenzoic acid	79.1	78.4
p-Hydroxybenzoic acid	30.2	40.5
L-Ascorbic acid	19.9	100
Potassium cyanide	80.6	95.8
Maleic hydrazide	4.3	4.8
EDTA	0	0
Citric acid	3.2	11.8
Sodium metabisulphite	100	100
Sodium sulphite	5.2	17.7
Sodium benzoate	15.2	19.1
p-Chloromercuribenzoate	19.6	22.1
2-Mercaptoethanol	99.1	99.6
Sodium azide	10.1	14.3
L-Cysteine	100	100
NaCl	0	0

116000 and 128000 [30–32]. Lewis and Dawson [33] also reported a MW of 100000 for the highly purified enzyme from *Lactarius piperatus*. Harel and Mayer [34] reported MW's of 30000–40000, 60000–70000 and 120000–130000 for the three interconvertible forms of *o*-diphenolases which they isolated from apple fruits. Although multiplicity of MW's appears to be a common feature of numerous *o*-diphenolases from various sources [14] the *D. rotundata* enzyme and that from *D. bulbifera* [13] seem to exist in a monomolecular form with a single MW. Considering that *o*-diphenolases include many groups of enzymes with differing substrate specificities, it is not unusual to observe such a wide spectrum of MW's and multiple molecular forms.

Copper content

The copper content of *D. rotundata o*-diphenolase was found to be 0.22%. This is very similar to the value of 0.21% obtained by Bouchilloux *et al.* [30] for mushroom *o*-diphenolase of MW 119000. Chaplin [24], however, obtained a significantly different value of 0.42% for a particulate preparation of *o*-diphenolase from cocoa husk. Based on an estimated MW of 107000 ± 5400 for our enzyme, a value of 0.22% for copper corresponds to *ca* four copper atoms per molecule of enzyme protein. Although the distribution of the copper atoms within the enzyme molecule is not yet certain, numerous reports suggest a copper content of one atom per polypeptide chain or subunit [32, 35, 36]. Other divergent views on the distribution and arrangement of copper in the functionally active enzyme exist [14]. The meagre work on the subunit structure of plant *o*-diphenolases and the possibility that some copper could be lost during purification [32, 37–39] add to the difficulty in arriving at an agreement on the copper content and distribution in this enzyme.

UV and IR absorption

The UV spectrum of the purified *D. rotundata o*-diphenolase exhibited a typical protein absorption spectrum with high absorptions around 215 nm attributed to the peptide linkage and a significant shoulder at 280 nm ($E^{1\%}_{1\,cm} = 14.2$) due to aromatic amino acids. There was no significant absorption above 300 nm. In general, the spectrum is similar to that reported for *o*-diphenolase from mushrooms [32] and *Solanum melongena* [26], except that the shoulder was observed exactly at 280 nm instead of 290 and 285 nm for the mushroom and *S. melongena* enzymes, respectively. Royal Ann cherry *o*-diphenolase also showed absorption maximum at 280 nm [6]. Unlike the enzyme from potato tuber [4] and tea leaves [25], aqueous solutions of *D. rotundata o*-diphenolase showed no colour. Apart from absorption around 280 nm, the enzymes from potato tuber and tea leaves absorbed in the region between 320 and 380 nm and at 611 nm, respectively.

The IR analysis gave peaks characteristic of proteins (3400–3300 cm^{-1}; 1650 cm^{-1}) and additional peaks around 1080 and 2100 cm^{-1} presumably duc to the C–O stretching bending and rocking vibrations and the NH stretching vibrations, respectively. This is similar to the IR spectrum reported in ref. [24] for the cocoa husk enzyme.

Effect of pH

The purified enzyme gave a bell-shaped pH-activity profile consisting of a single but broad pH curve between pH 5.5 and 7.0 with a maximum at pH 6.8, while most of the crude preparations from the various cultivars we worked with (Table 1) displayed two distinct pH optima. The pH optimum of most of the catechol oxidases studied is between pH 5.0 and 7.0. *o*-Diphenolases with a single pH optimum around neutrality have been isolated from various sources: peaches [5], cranberry [40], bean leaf [41], egg plant [42] and *D. bulbifera* [13]. A feature of the pH-activity profile which recurs in many reports is the presence of two peaks, a peak and a prominent shoulder [43–45] or a wide pH optimum [46–48]. It would appear therefore that the structure of the pH–activity relationship for *o*-diphenolases is dependent mainly on the source of the enzyme.

Plots of pH against V_{max}, V_{max}/K_m and log K_m and subsequent analysis by the method of Dixon and Webb [49] revealed groups with pK_a values of 4.7 and 7.2 involved in enzyme catalysis with catechol acting as substrate. Anosike and Ayaebene [50] using a similar approach implicated two groups with pK_a values of 3.8 and 7.4 in the activity of *D. bulbifera* polyphenol oxidase on catechol.

Temperature and enzyme stability

The optimum temperature for the *D. rotundata o*-diphenolase reaction was found to be 25°, although the enzyme retained its stability after incubation for 5–10 min at any temperature up to 60°. At 35, 50 and 60°, the enzyme retained 100, 98 and 96% of its original activity, respectively. Beyond 60° the activity dropped sharply with 20, 5 and 0% of original activity remaining at 80, 90 and 100°, respectively. The heat of inactivation (ΔH) estimated from an Arrhenius plot was 26.7 kcal. As suspected, the purified enzyme preparation was most stable when frozen maintaining almost 100% of its initial activity for over 2 weeks in this state. However, storage at room temperature resulted in loss of *ca* 80% of its activity in two weeks while at 4° the enzyme retained up to 90% of its activity during the same storage period. In view of the importance of *o*-diphenolase in yam tuber browning, knowledge of the stability and behaviour of this enzyme in the intact tuber during postharvest storage should be of interest to the food industry.

EXPERIMENTAL

Materials. The yams *D. rotundata, D. alata, D. cayenensis* and *D. bulbifera* and their local cultivars were from the 1980 crop purchased from markets in different parts of Nigeria.

Chemicals. Sephadex G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia. The enzyme substrates, catechol, DL-3,4-dihydroxyphenylalanine (DOPA), chlorogenic acid and pyrogallol and the marker proteins, egg albumen, cytochrome *c* and haemoglobin were from BDH. *p*-Cresol, tyrosine, *p*-coumaric acid, quinol and 3,4,5-trihydroxybenzoic acid were purchased from Hopkins and Williams. Catalase, bovine serum albumin, and D-(−)-lactate dehydrogenase were obtained from Sigma. Electrophoresis chemicals were from Shandon. All other chemicals used were of analytical grade.

Acetone powder preparation. The tubers of each of the 10 cultivars studied were peeled and cut into small pieces. The pieces (300 g) were soaked in 350 ml cold Me_2CO for 24 hr in the cold and the mixture marcerated in a Waring blendor. The resulting homogenate was filtered and the residue blended again with 900 ml cold Me_2CO and similarly filtered. The residue was spread out on sheets of aluminium foil and left to dry in the cold. The dry residue, known as the Me_2CO powder was stored in the freezer and used for preparing crude enzyme extracts either for further study, as they are, or for subsequent purification.

Crude enzyme preparation from acetone powder. Me_2CO powder (10 g) from each cultivar was suspended in 30 ml 0.1 M KPi buffer, pH 6.8, allowed to stand in an ice bath for 1 hr with constant stirring and then centrifuged at 20000 *g* for 10 min at 4°. The resulting supernatant was the crude enzyme.

Enzyme purification. Crude preparation from acetone powder. Crude enzyme extract was prepared from 80 g Me_2CO powder from *D. rotundata* (cv Zaria) with 240 ml KPi buffer as described above. *Ammonium sulphate precipitation.* Preliminary work had shown that most of the enzyme activity was pptated between 20 and 85% $(NH_4)_2SO_4$ satn. Therefore enough solid $(NH_4)_2SO_4$ was added to the crude enzyme soln from the previous step to achieve 20% satn and the mixture was stirred for 30 min at 4°. The resulting ppt was centrifuged at 25000 *g* for 10 min at 4°. The supernatant was then raised to 85% satn by addition of more solid $(NH_4)_2SO_4$, stirred and finally centrifuged as before. The pptated protein was redissolved in a minimal vol. of 0.1 M KPi buffer (pH 6.8) and the soln was subsequently dialysed for 2 hr at 4° against the same buffer. *Ion exchange chromatography.* The dialysed extract was applied to a DEAE–Sephadex A-50 column (2.6 × 40 cm) pre-equilibrated with 0.1 M KPi buffer, pH 6.8. The column was eluted with a linear gradient of 250 ml 0.1 M KPi buffer, pH 6.8 and 250 ml 0.5 M NaCl dissolved in the same buffer. 10 ml fractions were collected and assayed for both *o*-diphenolase activity and protein content. *Gel filtration.* Fractions (11–19) from the ion exchange step which showed high enzyme activity were pooled and concd to 19 ml against dry Sephadex G-100 particles and applied to a column of Sephadex G-200 (2.6 × 40 cm) pre-equilibrated with 0.1 M KPi buffer, pH 6.8. Elution was with the same buffer and 3 ml fractions were collected and assayed for activity and protein. The fractions that showed sufficient activity (23–35) were pooled and concd to 8 ml as mentioned before and then used for a second ion exchange chromatography step. *Second ion exchange chromatography.* 4 ml of the concd enzyme soln from the above step was applied to a DEAE–Sephadex A-50 column (2.2 × 22 cm) pre-equilibrated with 0.1 M KPi buffer, pH 6.8, and eluted with a linear gradient of 150 ml 0.1 M KPi buffer pH 6.8 and 150 ml 0.25 M soln of the same buffer. 4-ml fractions were collected and assayed for both enzyme activity and protein content. Active fractions were pooled, concd further and used for subsequent studies.

Assay of enzyme activity. The activity of *o*-diphenolase on catechol as well as on other substrates was assayed by a spectrophotometric determination of the initial rate of formation of the respective products at the proper wavelengths [51] according to ref. [24]. 1 unit of enzyme activity was defined as the amount of enzyme that caused a 0.001 change in *A* per min at the appropriate wavelength.

Protein determination. The protein contents of enzyme preparations were determined by the method of ref. [52] as modified in ref. [53] using BSA as standard.

PAGE. This was performed according to the anodic system of ref. [54] using tubes of 0.5 × 7.5 cm with 7.5% polyacrylamide in 25 mM Tris–HCl, pH 9. The gels were run at 5 mA/tube for 1.5 hr. After the run, three of the gels were stained for protein with 0.25% Coomassie brilliant blue in MeOH–HOAc–H_2O (5:1:5) while the other three gels were immersed in a soln of 30 mM catechol in 0.1 M KPi buffer pH 6.8 containing 0.05% *p*-phenylenediamine for 1 hr to stain for enzyme activity.

Copper determination. Cu was determined by atomic absorption spectrophotometry essentially as described in ref. [55] using $Cu(NO_3)_2 \cdot 3H_2O$ as standard.

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