The Peroxidase of *Dioscorea esculenta:* Partial Purification and Characterisation

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We isolated two types of peroxidase from the fresh tuber of *Dioscorea esculenta* using a combination of ion exchange and gel filtration chromatography. The results showed one type to be neutral and the other to be strongly ionic. The strongly ionic type constitutes 70% of total peroxidase activity in the tissue. The apparent molecular weight of the neutral type is 38 kDa while the anionic type has an apparent molecular weight of 57 kDa. It was possible with the use of gel filtration on Sephadex G-200 followed by FPLC on phenyl superose to purify the lower size POD by a factor of 15, while the larger ionic peroxidase was purified 68 fold compared to the crude with protein yields of 0.90% and 1.30% respectively. The ionic POD is more thermo-stable, has a higher optimum temperature for activity and has a higher apparent activation energy compared to the neutral POD from this source.

Introduction

Peroxidase (donor:H_2O_2 oxidoreductase, EC, 1:11:1:7) catalyses the oxidation of a wide range of physiological organic compounds by hydrogen peroxide. They are widely distributed in both plant and animal cells. Plants may be categorised into two broad types based on their peroxidase (POD) composition; viz. those that have powerful peroxidase and those with weaker peroxidase activities. When plants are injured, those with powerful peroxidase activities darken less rapidly (Hartree, 1955). The enzyme has been associated with diverse cellular functions including the changes in flavour and colour of raw under-blanched vegetables (Lee *et al.*, 1984, Vamos-vigyazo, 1981). The peroxidase of yams (*Dioscorea* spp.) has been largely neglected since the major post-harvest browning of yams has been strongly associated with the activity of yam polyphenol oxidase (PPO) by many workers (Anosike and Ikediobi, 1985, Bachem *et al.*, 1994). Thus, the focus of research in this regard has been on yam polyphenol oxidase (PPO). We have reported previously that some level of yam browning occurred outside the activity range of PPO (Omidiji and Okpuzor, 1996). Moreover, we obtained a slightly pure POD preparation with potential for PPO activity from the tissues of *D. esculenta* (Okpuzor and Omidiji, 1998). Accordingly, we wish to pay some attention to the POD from yams as a prelude to ascertain the role of this enzyme in the post-bruise browning of yams. This study focuses on the characterisation and purification of POD from *D. esculenta*; a popular yam type in Nigeria.

Materials and Methods

Enzyme source and POD assay

The tubers of *Dioscorea esculenta* were obtained and sustained as previously described (Omidiji and Okpuzor, 1996).

Tubers of *D. esculenta* were peeled and 100 g portion was homogenised in 400 ml of cold 0.05 M potassium phosphate buffer, pH 7.0 supplemented with 1.5 ml of Triton-X-100 in a Waring blender. The homogenate was centrifuged at 3,000×*g*. in the cold room for 20 min. The supernatant was flushed through a short column packed with 23 g. Bio-Rad SM-2 beads. The column was drained dry overnight to recover the enzyme. Peroxidase activity in the extract was determined according to Mujer *et al.* (1983) using O-dianisidine as substrate. One unit of enzyme activity is expressed as the amount of enzyme that caused a change of 0.001 absorbance unit per second under the assay condition. The protein content was determined according to Lowry *et al.* (1951).

Enzyme purification

A glass column (2×46 cm) was packed with soaked QAE-Sephadex A-50 resin (Pharmacia

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Fine Chemicals, Sweden; bed volume = 120 ml) in potassium-PO_4 buffer, 0.05 M, pH 7.0 and equilibrated with same buffer by washing until effluent attained a pH of 7.0. Final washing was at a flow rate of 0.5 ml min^{-1}. The enzyme extract was loaded onto the column and eluted with 300 ml of plain buffer followed by equal 50 ml buffer containing 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 M NaCl; 5 ml samples were collected at the washing flow rate. Eluted samples were assayed for POD activity as described and indirectly for protein by measuring absorbance at 280 nm. Enzymatically active fractions were pooled, concentrated by vacuum evaporation and subjected to gel filtration.

The concentrated fractions from ion exchange step (5 ml) were separately loaded onto a Sephadex G-200 column (2.5×40 cm, bed volume = 100 ml) pre-washed with the eluting buffer (potassium-PO_4 , 0.05 M, pH 7.0). Elution was at the rate of 0.3 ml min^{-1} and 3 ml samples were collected for POD and protein assays. Active fractions were pooled and concentrated in an Amicon Concentrator (Amicon Company, USA; molecular weight cut off = 10 kDa).

FPLC was carried out on pre-packed phenyl superose column designed for high performance hydrophobic chromatography. All the solutions used were degassed and filtered through 0.22 μm sterile Millipore filters. The enzyme fraction from gel filtration (0.1 ml) was injected into the column and 2 ml fractions were collected at the flow through rate of 1 ml min^{-1}. Protein absorbance was monitored automatically at flow-through while POD activity in the collected fractions were assayed as previously described.

Molecular weight determination

The molecular weight of the two peroxidases were determined on standard Sephadex G-200 column as described previously using aldolase (158 kDa), bovine serum albumin (BSA, 66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and cytochrome oxidase (12.4 kDa) as protein markers (Omidiji and Braimoh, 1994). The void volume of the column was determined by running blue dextran (2,000 kDa) though it at the same elution rate. The concentrated enzyme fractions (5 ml) from the gel filtration stage were eluted through the column at the same rate as the standard proteins and the molecular weight was calculated from a plot of partition coefficient (Kav) against the log. of molecular weight.

Enzyme characterisation

This was carried out as described by Simpson *et al.* (1987), using the two enzyme fractions from the gel filtration stage. The substrate (O-dianisidine) was incubated in 0.05 M phosphate buffer, pH 7.0 with regular stirring at the desired temperature (10–70 °C) until the equilibration temperature was attained (usually 3–5 min). The enzyme which was maintained at room temperature

(29 ± 2 °C), was then introduced and the assay was carried out as described.

The stability of the enzyme fractions in various heat regimes was assayed as described by Owusu-Ansah (1989) within a temperature range of 35–75 °C.

Double reciprocal (Arrhenius) plots for substrate-enzyme reaction at different temperatures (25–55 °C) were obtained and used to calculate the apparent activation energy (Ea) as described by Morris, (1968).

The substrates were used at 10 mM being the optimum concentration for most of the compounds as previously determined (Omidiji and Okpuzor, 1996). They were pre-dissolved in the buffer and the enzyme activity determined as usual.

The inhibitors were employed at 5 mM; this being the concentration at which the classical POD inhibitor (DTT) showed the maximum effect as previously determined (Omidiji and Okpuzor, 1996). The inhibitors were added to the substrate-in-buffer, incubated for 5 min before the addition of the enzyme followed by enzyme assay as usual.

Results and Discussion

The SM-2 beads used to remove Triton X-100 slightly improved purification by a factor of 2.0 with a protein yield of 66%. This suggests that either the SM-2 beads unmasked some inhibition of the enzyme or directly activated the enzyme. Kenten (1957) had extracted a phenolase inhibitor complex from a crude extract of broad beans. Zucker (1972) suggested that the control of peroxidase activity could be effected through the activation of pre-formed inactive enzyme molecules. The effect has been suggested to be mediated through

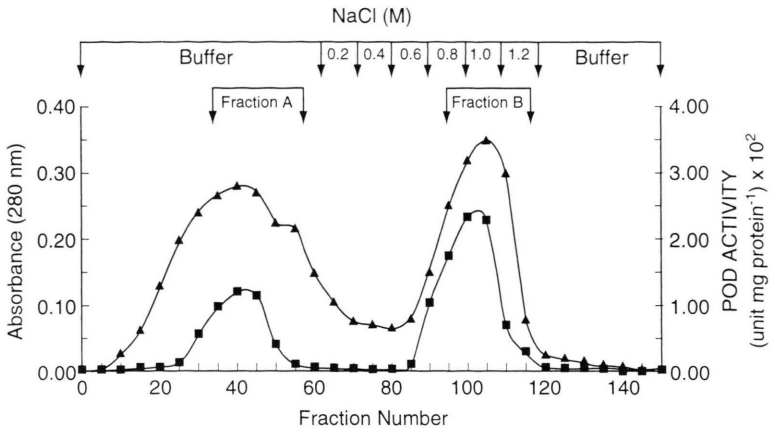

Fig. 1. The elution profile of crude extract of *Dioscorea esculenta* on QAE-Sephadex A-50. The column (2.5×46 cm, bed volume = 120 ml) was washed with eluting buffer (Potassium-PO_4, 0.05 M, pH 7.0) until clean. The crude extract was then loaded and eluted with 300 ml of washing buffer. Further elution was with 50 ml portions of the same buffer containing 0.2–1.2 M NaCl at the rate of 0.5 ml min^{-1}; 5 ml fractions were collected. (▲), protein content; (■), peroxidase activity.

a phenol-peroxidase interaction during extraction (Fieldes and Tyson, 1973).

The elution profile of the crude extract on QAE-Sephadex A-50 is shown in Fig. 1. The neutral peroxidase which eluted through the column unbound constitute 30% while the remaining 70% POD tissue activity was associated with the ionic fraction B which eluted with 1.0 M NaCl. This pattern is similar to what we observed earlier with yam polyphenol oxidase (Okpuzor and Omidiji, 1998). The elution of the two PODs on gel filtration clearly resolved the two peroxidase types. When the two fractions were combined in equal proportions and subjected to gel filtration, two clear peaks were obtained with median points at fraction numbers 22 and 98 for POD A and B respectively. Thus it is correct to suggest that there are two the major POD types in this yam tissue; the predominant type being strongly ionic. Fraction A has an apparent molecular weight of 38 kDa and is thus a typical POD while fraction B has an apparent molecular weight of 57 kDa and is also a typical POD (Vamos-Vigyazo, 1981).

The purification profile through to the FPLC stage is shown in Table I. We were able to purify the predominant POD by a factor of 68 while the neutral POD was purified 15-fold. These purification factors are comparable to the values obtained by De Baisi and Badiani, (1990) for the leaves of *Triticum aestivum* using FPLC. The relatively low purification could be due to the slimy nature of yam extracts which makes it essential for the ion exchange step to precede the gel filtration.

Some physico-chemical properties of the two enzymes are presented in Table II. The strongly ionic POD has higher optimum temperature, apparent activation energy and it is more thermostable. We hereby suggest that it is a more stable

Table I. The purification profile of two peroxidases from *D. esculenta*.

Step	Volume [ml]	Enzyme activity [unit ml^{-1}]	Total activity [unit]	Protein concentration [mg ml^{-1}]	Total protein [mg]	Specific activity [unit mg protein^{-1}]	Purification factor [unit]	Protein yield [%]
Crude	160	240	38400	2.30	368.0	104	1.00	100.00
SM 2 beads	255	200	51000	0.95	242.3	211	2.02	65.83
QAE-Sephadex A-50 (Fraction A)	125	40	5000	0.08	10.00	500	4.79	2.72
QAE-Sephadex A-50 (Fraction B)	150	120	18000	0.15	22.50	800	7.67	6.11
Gel filtration (fraction A)	45	165	7425	0.25	11.25	660	6.33	3.06
Gel filtration (fraction B)	120	250	30000	0.12	14.40	2083	19.97	3.91
FPLC (fraction A)	6	830	4980	0.55	3.30	1509	14.46	0.90
FPLC (fraction B)	16	2125	34000	0.30	4.80	7083	67.88	1.30

Table II. Some physico-chemical properties of two peroxidases from *D. esculenta*.

Property	Neutral peroxidase (fraction A)	Ionic peroxidase (fraction B)
Molecular weight (kDa)	38.0 ± 2.8	57.0 ± 4.6
Optimum pH	6.0 and 7.5	6.0 and 7.5
Optimum temperature (°C)	47.4 ± 1.5	55.2 ± 2.2
Thermostability (residual activity after 60 min at 70 °C (% control)*	25.6 ± 4.2	35.2 ± 5.1
Apparent activation energy (Ea, kJ mol^{-1})	101.3 ± 27.4	361.5 ± 39.8

* Control (100%) activity was the activity of the ionic fraction at room temperature (32 ± 2 °C) in the presence of 10 mM O-dianisidine. This activity was 3,250 ± 68 units mg $protein^{-1}$ under the assay condition. Each reading is the mean of two determinations.

Table IV. The Inhibitors of yam peroxidase.

Compound/Inhibitor	% Inhibition of control Neutral POD (A)	Ionic POD (B)
O-Dianisidine (control substrate, no inhibitor)	0	0
Benzoic acid	0	0
Gallic acid	5	0
Sodium azide	20	11
Potassium cyanide	22	19
L-cysteine	44	30
Potassium fluoride	38	33
Hydroquinone	55	35
Citric acid	32	38
Polyethylene glycol	46	50
Thioglycollate	56	79
Iodobenzoic acid	60	64
Dithiothreitol	98	100

Control activity is enzyme activity at room temperature in the presence of O-dianisidine without any inhibitor. The control value was 2,781 ± 58 units mg $protein^{-1}$. Each reading is the mean of two assays. The reaction mixture contained 5 mM inhibitor which was incubated with the mixture 5 min before the addition of the normal substrate (O-dianisidine). The substrate concentration was 10 mM in 0.05 M potassium phosphate at pH 7.0.

form of the enzyme in the tissues and probably represents the more functional form of the enzyme in the tissue, (Miller *et al.*, 1990; Ling and Lund, 1978).

Tables III and IV show the substrate and inhibitor characteristics of the neutral and ionic peroxidase from *D. esculenta*. Generally, the predominant and ionic POD is more active with the substrates used compared to the neutral one, while it is also less sensitive to the inhibitors. The results show that the two enzymes are active PODs as shown by complete inhibition by DTT (Okpuzor and Omidiji, 1998; Loomis and Battaile, 1966).

We hereby provide evidence for the existence of two PODs in the tissues of *D. esculenta* and

Table III. The effect of some substrates on the activity of two peroxidases from *D. esculenta*.

Substrates		Enzyme activity (% of Control) Neutral POD (A)	(% of Control) Ionic POD (B)
Amines	O-dianisidine (control)	89	100
	di-amino benzidine	2	9
	p-amino phenol	15	20
	p-phenylene diamine-HCl	45	41
	p-amino benzoic acid	52	57
	benzidine	58	80
Monophenol	*p*-cresol	0	0
	L-tyrosine	0	0
Diphenol	4-methylcatechol	5	12
	resorcinol	35	44
	catechol	50	55
Polyphenol	phloroglucinol	35	44
	Pyrogallol	56	72

The control activity is the enzyme activity of the ionic peroxidase at room temperature in the presence of O-dianisidine. This enzyme activity was 3,350 ± 50 units mg $protein^{-1}$ under the assay condition (mean of two assays).

present some biochemical properties of the two enzymes. Peroxidase activity is predominantly ionic in this tissue. We will proceed to investigate the role of these enzymes in the post-harvest spoilage of yam tubers, while at the same time we will attempt to purify the enzymes to homogeneity.

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