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Characterization of Peroxidase in Plant Cells[1]

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ABSTRACT

Two peroxidases, one anionic and one cationic, have been purified from the proteins secreted by peanut (*Arachis hypogaea* L. var Virginia 56R) cells in suspension culture. These two peroxidases apparently have identical catalytic properties.

A definitive role for peroxidases (EC 1.11.1.7) in plants has eluded plant scientists so far. There have been numerous reports in the literature with respect to their general involvement in lignin synthesis (3, 4) and oxidation of the endogenous IAA (7). A trend has emerged in the last few years, where lignin synthesis has been associated with the anionic peroxidases (1, 3) and the oxidation of IAA with the cationic peroxidase isoenzymes (1, 8). Little attention has been paid in the purification of peroxidase to a homogenous protein preparation (5, 8).

In an attempt to assign a functional role to the two groups of peroxidase, the anionic and cationic forms have been purified from the medium of peanut cells grown in suspension culture (19).

These two proteins with peroxidative activity have been purified to apparent homogeneity and tested with regards to their structure, mol wt, and specific activities with different substrates. It was noted that the two molecules differed with respect to their structure and mol wt, but not in their catalytic properties.

MATERIALS AND METHODS

Peanut (*Arachis hypogaea* L. var Virginia 56R) cells in suspension cultures, derived from cotyledonary tissue were maintained in 14-d culture cycles as reported before (10).

Purification of the Cationic and Anionic Proteins with Peroxidase Activity. A crude preparation of proteins from the conditioned cell medium (2) was obtained by sequential precipitation with 70% acetone and 80% $(NH_4)_2SO_4$ as described (13). This crude preparation of proteins was then chromatographed over a CMC[2] column at pH 5.0. The anionic proteins, that did not bind to the CMC, were recovered in the flow-through fraction. The cationic peroxidase was isolated from CMC bound proteins as described (13).

The flow-through proteins were concentrated by precipitation with 80% $(NH_4)_2SO_4$ and dissolved in 0.005 M Tris-Cl buffer pH 8.4. These proteins were then dialyzed against 4 L of Tris-Cl buffer as above for 12 to 14 h. Next the dialysate was chromatographed over DEAE-Sepharose. The anionic peroxidase on this column was eluted with a gradient of 0 to 0.2 M NaCl in 0.005 M Tris-Cl, pH 8.4. The effluent fractions were monitored at 280 nm on an ISCO UA2 monitor. The Reinheitzahl (RZ) value ($A_{407}/A_{280\,nm}$) of the fractions was calculated. Fractions with RZ values exceeding 2 were pooled. These were concentrated by precipitating with 70% acetone and resolubilizing in 0.05 M phosphate buffer pH 7.0, for enzyme assays and other determinations. Protein determinations were made according to the method of Lowry *et al.* (12).

SDS Gel Electrophoresis. SDS-PAGE on 5 to 17.5% gradient gel was performed essentially according to the technique of Laemmli (11).

Sedimentation Analysis. The purified cationic and anionic proteins, dissolved in 0.05 M phosphate buffer pH 7.0, were analyzed in Beckman Model E Ultracentrifuge for sedimentation coefficient determination. The samples were spun at 50,740 rpm at 20 to 25°C in an AN-E rotor and photographs with Schlieren optics were taken at 16-min intervals. Sedimentation coefficients were calculated according to the method of Markham (14) and corrected to water at 20°C.

Enzyme Assays. Peroxidase assays were carried out on appropriately diluted solutions using one of the following substrates in a total volume of 3 ml. For 4-α-amino antipyrine the procedure used was essentially that outlined in Worthington enzyme manual (20). For the substrates, guaiacol and eugenol, the procedures have been outlined (16).

The IAA-oxidase assays were carried out by incubating the peroxidase (0.5–1 mg protein) with 10 μM IAA and the McGilvray citrate-phosphate buffer at varying pH values (2.6–8.0) in a total volume of 2 ml. After incubation for 30 min, the reaction was stopped by adding 4 ml of Salkowski reagent (6) and incubating in the dark for 30 min. The amount of residual IAA was determined by reading the absorption of the pink color that developed at 540 nm. The enzyme activity is expressed as μg IAA oxidized mg^{-1} protein min^{-1}.

RESULTS AND DISCUSSION

The results presented in Figure 1 clearly show that the anionic protein (channels a and f) and the cationic protein (channels d and e) have different mobilities in SDS PAGE. A double protein band (channel c) was observed when the anionic and cationic proteins were electrophoresed together in one sample thus inferring a small difference in mol wt for the two proteins. This is a confirmation of an earlier observation (17). The cationic and anionic proteins exhibited RZ values of 3.6 and 2.0, respectively. This is in contrast to the observations made for horse radish peroxidase where higher RZ values were observed for the anionic fraction (15). But the single protein band on SDS-PAGE and a single peak in ultracentrifugal analysis (Fig. 2) show the homogeneity of the preparation of anionic peanut peroxidase. Thus this implies that RZ value alone based on heme absorption at 407 nm cannot be taken as a measure of purity, since it depends equally on the aromatic amino acid composition of each protein (21).

With the apparent purity of the proteins established, the main objective of this experiment was to assay whether differences in

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[2] CMC, carboxy methyl cellulose; RZ, Reinheitzahl.

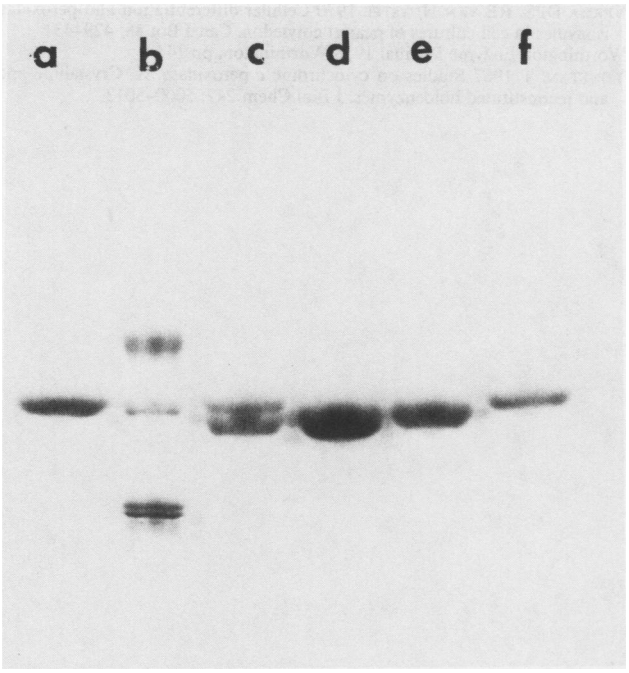

FIG. 1. Determination of mol wt of anionic and cationic peroxidase from peanut cells. Electrophoresis was carried out on a 5 to 17.5% gradient slab gel. Two concentrations for anionic (a, 20 μg and f, 10 μg) and for cationic (d, 40 μg and e, 25 μg) and 15 μg each for the combined sample (c) were used. Channel b carries marker proteins (serum albumin 67 kD; ovalbumin 43 kD; soybean trypsin inhibitor 20 kD and Cyt (12.5 kD). Following electrophoresis the gel was stained with Coomassie blue.

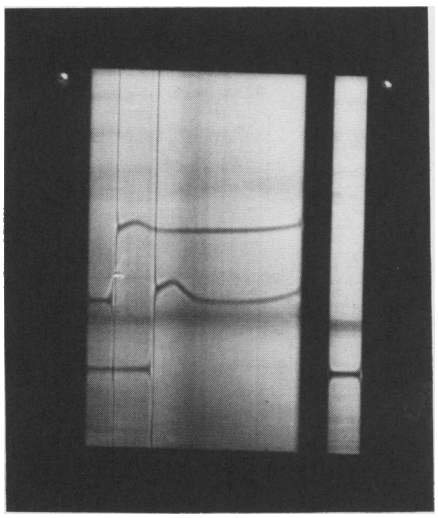

FIG. 2. Ultracentrifugal analysis of the anionic peroxidase (0.7 mg/ml) alone (top row) and the anionic and cationic peroxidases together at a final concentration of 1.0 and 0.5 mg/ml, respectively (bottom row). Schlieren pattern at 32 min of centrifugation. Sedimentation is towards right.

the various catalytic properties associated with each protein molecule did occur. The results presented in Table I show no major differences in peroxidase activity between the cationic and anionic proteins. This is particularly true for eugenol which is considered to be a natural substrate for detecting the capability of lignin synthesis. Even for assay with guaiacol, a lignin precursor as well the data are not highly different considering the SE. Furthermore, no difference could be observed in their capability for IAA-oxidation (Table I). Even the pH optima for the two

Table I. *Comparison of the Properties of the Anionic and Cationic Fractions of Peanut Peroxidase*

Property	Peroxidase Fraction	
	Anionic	Cationic
Mol wt	42kD	40kD
Sedimentation coefficient	3.5*s*	3.5*s*
Specific peroxidase activity[a]		
guaiacol[b]	907 ± 133	637 ± 30
eugenol[b]	37.0 ± 2.03	44.3 ± 6.4
α-aminoantipyrine[c]	78.4 ± 0.005	62.5 ± 3.77
IAA-oxidase activity		
pH optimum	3.6	3.6
specific activity[a] (μg IAA oxidized mg^{-1} protein min^{-1})	10 ± 0.25	12 ± 0.35
RZ (*A*407/280 nm)	2.0	3.6

[a] ± SE.

[b] Δ A units min^{-1} mg^{-1} protein

[c] $\text{Units} = \dfrac{\Delta A510 \text{ nm } min^{-1}}{6.58 \text{ } mg^{-1} \text{ enzyme } ml^{-1} \text{ reaction mixture}}$

proteins was the same. This similarity of two peroxidases with opposite charge is in disagreement with the results on horse radish peroxidase, where it was postulated that the anionic forms had more peroxidative activity and the cationic forms more oxidative capability, using oxalacetate oxidation (9). This is in contrast to views held by others (1).

In spite of the similarities in catalytic properties of these two peanut proteins, they differ in their tryptic cleavage patterns (17) and are not immunologically related (18).

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