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PEROXIDASE FROM *EUPHORBIA CHARACIAS* LATEX: PURIFICATION AND PROPERTIES

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Key Word Index—*Euphorbia characias*; Euphorbiaceae; peroxidase; hydrogen peroxide; hemoproteins.

Abstract—A peroxidase has been purified to homogeneity from *Euphorbia characias* latex using ammonium sulfate precipitation and chromatography on DEAE-cellulose, hydroxylapatite and SP-Sephadex columns. The substrate specificity of the enzyme is typical of a plant peroxidase except that it shows no activity with indole-3-acetic acid. The pH optimum of the enzyme was 5.75 and the isoelectric point 7.4. The activation energy was 14 kcal/mol. The prosthetic group was shown to be ferriprotoporphyrin IX. Gel chromatography and PAGE indicate that the purified protein is composed of a single polypeptide chain having a MW of *ca* 48 000.

INTRODUCTION

Peroxidases (peroxidase: EC 1.11.1.7. donor: H_2O_2 oxidoreductase) utilizes hydrogen peroxide or substituted peroxides for the oxidation of a large number of substrates. Peroxidases are widely distributed and have been isolated from many higher plants [1–9], animal tissues, yeasts and various micro-organisms [10]. The wide distribution of peroxidases suggest that they could be of great biological importance. Although many experimental studies have concerned the physico-chemical properties, reaction mechanism [11] and the nature of the reaction products with many organic compounds [12], the physiological functions and metabolic control of these enzymes are still poorly understood.

Another question is the hydrogen peroxide source. At present little is known about the distribution in higher plants of enzyme systems which lead to hydrogen peroxide production. These enzymes may be of importance not only because of the oxidations they catalyse, but also because the hydrogen peroxide formed can be used by peroxidase for coupled oxidations [13]. Kenten and Mann [14] showed that hydrogen peroxide is produced by enzyme systems present in extracts of many plants, and that it can be used by the extracts for the oxidation of Mn^{2+} [15, 16]. Attempts to identify these systems have so far shown that mono and diamines are oxidized by extracts of pea seedlings with the production of hydrogen peroxide [17, 18]. In later studies the diamine oxidase was isolated and characterized, and a new physiological role for these enzymes suggested [19]. Another source of hydrogen peroxide is represented by the activity of some peroxidases. In fact it has been shown that horseradish peroxidase [20] and tobacco peroxidase [21], in the presence of phenolic compounds and Mn^{2+} as cofactors, can catalyse the oxidation of NADH with the production of hydrogen peroxide. This hydrogen peroxide can be used for the peroxidatic polymerization of cinnamyl alcohols leading to lignin-like substances [22]. Thus, it appears that peroxidases can catalyse two different reactions for lignin biosynthesis: the production of hydrogen peroxide and the polymerization of cinnamyl alcohols.

Recently we have found diamine oxidase and peroxidase activity in *Euphorbia characias* latex. After isolating a diamine oxidase [23] we have attempted to purify the peroxidase in order to study *in vitro* the reconstituted coupled system.

We report in this paper the isolation and physicochemical characterization of the peroxidase from the latex of *Euphorbia characias* (EP).

RESULTS

Criteria of purity

EP obtained by the purification procedure summarized in Table 1 was tested for homogeneity by PAGE in different conditions. Only one protein band with enzymatic activity was present on PAGE and on analytical gel electrofocusing in the pH ranges 3–10 and 5–8. The isoelectric pH of EP is 7.4. Only one band was observed in SDS-PAGE in the presence and absence of mercaptoethanol. The presence of isoenzyme species was not detected by these analytical methods.

MW determination

The MW of EP was determined by SDS-PAGE and gel filtration on Sephadex G-200. SDS-PAGE showed a single band with a MW of 46 000 ± 1000. On gel filtration the protein was eluted with a K_d of 0.425, corresponding to a MW of 49 000.

Iron, heme and sugar content

The purified enzyme contains 0.12% of iron. On this basis a minimum MW of 46 500 may be calculated. The heme prosthetic group was identified as iron-protoporphyrin IX by the oxidized and reduced forms of the pyridine hemochromogen derivative. With a molar extinction coefficient of 34.2×10^3 for the 557 nm absorp-

Table 1. Purification of *Euphorbia* latex peroxidase (starting material was 300 ml latex)

Step	Total volume (ml)	Protein (mg/ml)	Sp. act. (units/mg)	Total act. (units)	Purification (fold)	Yield (%)
1. *Euphorbia* latex	300	26	0.24	1870	1	100
2. Acetone powder extract	775	4	0.587	1820	2.5	97
3. Dialysis against distilled water	765	3	0.78	1790	3.2	95
4. Ammonium sulfate fractionation	164	6.7	1.28	1400	5.3	74
5. DEAE-cellulose column	240	0.3	16.7	1200	69.5	64
6. Hydroxylapatite column	19	0.12	400	910	1670	49
7. SP-Sephadex column	9.4	0.107	840	840	3500	44

tion peak of the pyridine derivative of ferroprotoporphyrin IX, the minimal MW of EP was calculated to be 48 000.

The presence of 8% (w/w) neutral sugars was also found. The average MW obtained by the four methods reported, yielded a value of 47 000. The enzyme consists of a single polypeptide chain and contains one iron atom per mole.

Spectroscopic properties

Figure 1 shows the absorption spectra for the native and dithionite-reduced forms of EP. In the visible region the spectrum of the oxidized enzyme shows absorption maxima at 400, 494 and 633 nm. In Table 2 are listed the absorption maxima and the related millimolar extinction

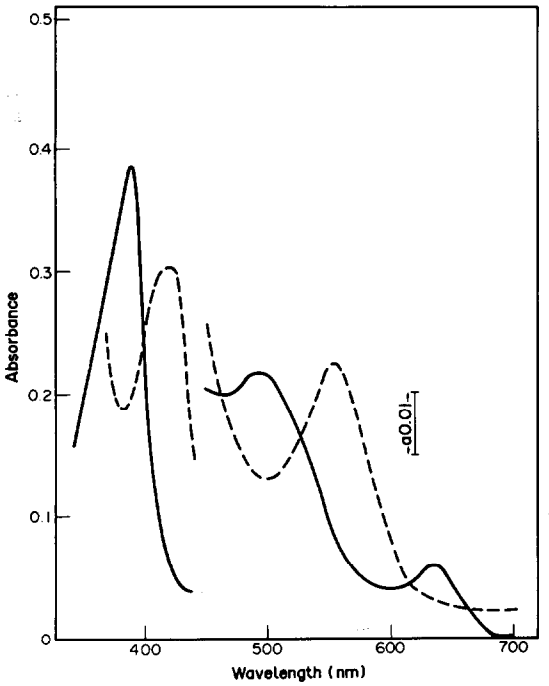

Fig. 1. Absorption spectra in the visible region of the native (——) and dithionite-reduced (– – –) forms of *Euphorbia* peroxidase (0.198 mg/ml) in 0.1 M potassium phosphate buffer pH 7.

Table 2. Summary of the spectral properties of *Euphorbia* peroxidase

Derivative	Absorption maxima (nm)	E^{mM}_{1cm}
Oxidized	400	95.8
	494	11.3
	633	3.5
Oxidized + cyanide	354	28.3
	418	95.7
	538	9.9
Reduced	430	74.6
	552	10.4
Reduced + cyanide	422	90.5
	533	10.7
	560	11.5
Reduced + carbon monoxide	420	153.7
	538	11.6
	566	10.4
Hydrogen peroxide	410	29.6

coefficients observed in the visible wavelength region for the oxidized enzyme and some of its derivatives, that is: reduced, cyanide, carbon monoxide, hydrogen peroxide. The absorption spectra are characteristic of a typical plant peroxidase [1–9], strongly suggesting that hematin is the prosthetic group of the enzyme. In the UV region the protein absorption peak is centred at 270 nm with $E^{M}_{1cm} = 70 \times 10^3$. The R_z value (A_{400}/A_{270}) of the purified peroxidase was 1.36.

Substrate specificity and inhibitors

In the presence of hydrogen peroxide EP catalyses the oxidation of many phenols and aromatic amines (Table 3). In this respect, its behavior is similar to that of all other known plant peroxidases. It is not possible to determine the true specificity of the enzyme because its natural substrates are unknown. It is worth noting that, at variance with other plant peroxidases, EP does not show any activity with IAA as substrate in the presence of dichlorophenol and manganese ions. This peculiar property may be indicative of a specific physiological role of the enzyme in this plant.

EP is inhibited by typical inhibitors of heme proteins,

Table 3. Substrate specificity of *Euphorbia* peroxidase

Substrate	Relative reaction rate
o-Dianisidine	100
Benzidine	12
Pyrogallol	40
o-Phenylenediamine	12
Ascorbate	12
DOPA	12
Orcinol	11
Resorcinol	11
Aniline	5
Indoleacetic acid	0

such as cyanide ($K_i = 10^{-5}$ M) and azide ($K_i = 3 \times 10^{-3}$ M), while the more specific metal chelators EDTA and DDC as well as the sulfhydryl group reagents are ineffective.

Effect of pH, buffer and temperature

The effect of pH on EP activity was tested in Na-acetate, potassium-phosphate and Tris–HCl buffers. As shown in Fig. 2, the pH curves show an optimum at 5.75 in 0.1 M Na-acetate buffer using *o*-dianisidine as substrate. The effect of temperature on the rate of the reaction was studied over the range 20–50° under standard assay conditions. A straight line was obtained in the Arrhenius plot with an activation energy of 14 kcal/mol.

EXPERIMENTAL

Chemicals. All the chemicals were obtained as pure commercial products and used without further purification. Standard proteins and DEAE-cellulose were from Serva; SP-Sephadex A50 and Sephadex G-200 (fine grade) from Pharmacia; hydroxylapatite (Bio-Gel HTP) from Bio-Rad.

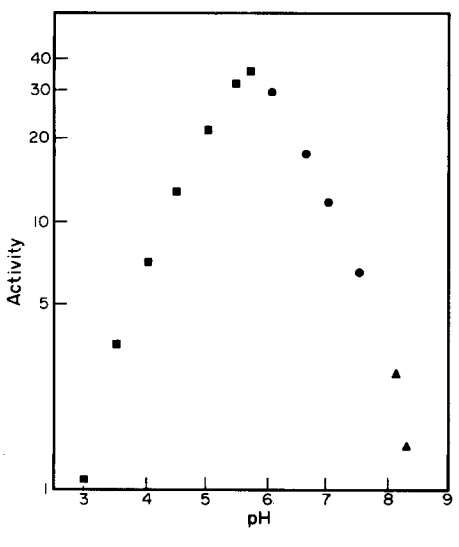

Fig. 2. Effect of pH and buffer composition on *Euphorbia* peroxidase activity. The Buffers used were: (■) 0.1 M Na acetate, (●) 0.1 M potassium phosphate and (▲) 0.1 M Tris–HCl at the indicated pH values.

Purification. The purification procedure follows up to step 4 the method described in ref. [23]. The supernatant (step 4) was made 10 mM with KPi-buffer pH 7 and loaded onto a DEAE-cellulose column (2.8 × 14 cm) equilibrated with the same buffer. In these conditions the enzyme was not bound and was collected with the washings until the *A* at 280 nm was below 0.01. The soln was dialysed against 30 l. of H_2O at 4° for 12 hr and the insoluble material removed by centrifugation. The supernatant (step 5) was loaded onto a Bio-Gel column (2.8 × 8 cm) equilibrated with 10 mM KPi-buffer, pH 7. The column was washed with 200 mM KPi-buffer, pH 7 (flow rate 100 ml/hr), until the eluate had no further *A* at 280 nm. The bound yellowish peroxidase was then eluted with 400 mM KPi-buffer, pH 7 (flow rate 100 ml/hr; 10 ml fractions). The fractions showing enzymatic activity were pooled and dialysed for 12 hr against 5 l. of 5 mM KPi-buffer, pH 7. The insoluble material was removed by centrifugation. The supernatant (step 6) was applied to a SP-Sephadex column (2 × 10 cm) equilibrated with 5 mM KPi-buffer pH 7 and washed with the same buffer until the eluate had no further *A* at 280 nm. The enzyme was eluted with 10 mM KPi-buffer, pH 7 (flow rate 100 ml/hr; 10 ml fractions). The fractions with the highest sp. act. were pooled and concentrated by ultradialysis (step 7). A summary of a typical purification is presented in Table 1. The overall purification achieved was 3500-fold with a recovery of *ca* 44%.

Enzyme assay. EP activity was tested by following the change in *A* at 460 nm due to *o*-dianisidine oxidation in the presence of H_2O_2 and enzyme [24]. The complete reaction mixture contained: 300 μmol of NaOAc. buffer, pH 5.75, 120 μmol of *o*-dianisidine or other substrates (see Table 3), 90 μmol of H_2O_2 and enzyme in a final vol. of 3 ml. The mixture was incubated at 25° and the reaction was started by the addition of H_2O_2. Protein bands with activity were detected on PAGE and isoelectric focussing by staining the gel after the electrophoretic run in 5 ml of 0.1 M NaOAc buffer, pH 5.75 containing 200 μmol of *o*-dianisidine and 150 μmol of H_2O_2. Unit of activity was defined as the amount of enzyme required to produce a change in *A* of 0.1 at 460 nm per min. Sp. act. was defined as units of activity per mg of protein. The indole-3-acetic acid oxidase activity was measured as reported in ref. [25].

Analytical PAGE. Electrophoresis under non-denaturing conditions was performed as described in ref. [26]. The isoelectric point of EP was measured by PAGE in the pH range of 3–10 and 5–8, as described in ref. [27]. Continuous SDS-PAGE was carried out according to ref. [28]. For MW determination, proteins with the following MW were used as standard: lysozyme (14 300), soybean trypsin inhibitor (21 000), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000) and phosphorylase B (94 000).

Estimation of MW by gel filtration. Determination of MW under non-denaturing conditions was performed by gel filtration at 4° using a column (2.5 × 90 cm) of Sephadex G-200 (fine grade) equilibrated and eluted with 0.1 M KPi-buffer, pH 7, containing 0.3 M KCl (flow rate 14 ml/hr; 3 ml fractions). The distribution coefficient (K_d) was obtained as described in ref. [29] using blue-dextran to measure the void vol. (V_o) and tyrosine to measure the total vol. (V_i). The standard proteins used and their MWs were: ovalbumin (43 000), bovine serum albumin (67 000), aldolase (140 000) and catalase (240 000).

Iron determination was performed by atomic absorption using an IL 951 atomic absorption spectrophotometer equipped with a graphite furnace. The spectral line chosen was 2483 Å.

Other analytical methods. Protein concn was estimated by the

method of ref. [30], using crystalline bovine serum albumin as standard. Neutral sugars were estimated by the phenol–H_2SO_4 method [31] using D-glucose, D-galactose and D-mannose as standards. Heme concn was determined according to ref. [32].

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