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PEROXIDASE FROM *IPOMOEA BATATAS* SEEDLINGS: PURIFICATION AND PROPERTIES

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Key Word Index—*Ipomoea batatas*; Convolvulaceae; peroxidase; hydrogen peroxide; plant hemoprotein.

Abstract—A peroxidase has been purified to homogeneity from *Ipomoea batatas* seedlings using ammonium sulphate precipitation and chromatography on DEAE-cellulose and SP-Sephadex columns. The pH optimum of the enzyme was found to be dependent on the buffer and substrate used. The isoelectric point is 7.3. The activation energy was estimated to be 14 kcal/mole. The prosthetic group was shown to be ferriprotoporphyrin IX. Gel chromatography and PAGE indicate that the purified protein is composed of a single polypeptide of MW 42 000. The amino acid composition appears to be similar to those reported for other plant peroxidases.

INTRODUCTION

Peroxidases (peroxidase: EC 1.11.1.7. Donor, H_2O_2 oxidoreductase) are widely distributed and have been isolated from many higher plants [1–10], animal tissues, yeasts and various micro-organisms [11]. The wide distribution of the enzyme suggests that it could be of great biological importance. However the role that it plays in metabolism is not clear due to the large number of reactions it catalyses and to the considerable number of isoenzymic species. In fact peroxidase isoenzymes occur in plants either bound to the cell-wall [12] or located in the protoplasts [13]. Cell-wall bound peroxidases are probably involved in lignification [14] while other isoenzymes may have a regulatory role in plant senescence [15] or in the destruction of auxins [16, 17].

This paper describes a simple method for isolating a new peroxidase (IP) from *Ipomoea batatas* seedlings. Isoenzymes were not detected. Some molecular and kinetic properties of the purified enzyme are given.

RESULTS

Criteria of purity

IP obtained by the purification procedure (Table 1) was tested for homogeneity by PAGE under different conditions. Only one band with enzymic activity was present both on PAGE and on analytical gel electrofocusing in the pH ranges 3–10 and 5–8. The isoelectric pH of IP is 7.3. Only one band was observed in SDS-PAGE in the presence and absence of mercaptoethanol.

MW determination

The MW of IP was determined by SDS-PAGE and gel-filtration on Sephadex G-200. SDS-PAGE showed a single band with a MW of 41 000 ± 1000. On gel filtration the protein was eluted with a K_d of 0.450, corresponding to a MW of 42 000.

Iron, heme and sugar content

The purified IP contains 0.11% Fe. On this basis a minimum MW of 42 600 may be calculated. The heme prosthetic group was identified as iron protoporphyrin IX through the oxidized and reduced forms of the pyridine-hemochromogen derivatives. With an ε value of 34.2 $\times 10^{-3}$ for the 557 nm absorption peak of the pyridine derivative of ferro-protoporphyrin IX, the minimal MW of IP was calculated to be 41 300. The presence of 18% (w/w) neutral sugars was also found.

Spectroscopic properties

In the visible region the spectrum of the oxidized enzyme shows absorption maxima at 400, 490 and 633 nm with $E^{M}_{1cm} = 90 \times 10^3$, 10×10^3 and 3×10^3 respectively.

Table 1. Purification of *Ipomoea batatas* seedling peroxidase

Step	Total protein (mg)	Sp. act. (units/mg)	Total act. (units)	Purification (fold)	Recovery (%)
1. Crude homogenate	34 500	62.4	2 154 000	1	100
2. Ammonium sulphate fractionation	3615	268	969 000	43	45
3. DEAE-cellulose column	2	420 000	840 000	6730	39
4. SP-Sephadex column	0.9	660 000	600 000	10 500	27.8

In the UV region the protein absorption peak is centred at 270 nm with $E^{M}_{cm} = 63 \times 10^3$. The R_z value (A_{400}/A_{270}) of the purified peroxidase was 1.8.

Substrate specificity and inhibitors

In the presence of hydrogen peroxide, IP catalyses the oxidation of many phenols and aromatic amines. In this respect, its behaviour is similar to that of other plant peroxidases. The most rapidly oxidized substrate is *o*-dianisidine, while pyrogallol, benzidine, *o*-phenylendiamine, ascorbate, orcinol and resorcinol are oxidized with decreasing rates. In the presence of dichlorophenol and manganese ions, IP shows IAA activity ($K_m = 1.25 \times 10^{-5}$), at variance with *Euphorbia* peroxidase [10], but similar to the majority of other plant enzymes of this class. The enzyme is inhibited by typical inhibitors of heme proteins, such as cyanide (K_i: 5×10^{-7} M) and azide (K_i: 2×10^{-3} M), while sulphydryl group reagents are ineffective towards peroxidase activity.

Effect of pH, buffer and temperature

The effect of pH on peroxidase activity was tested in NaOAc, K-Pi and Tris–HCl buffers. The pH curve shows an optimum at 5.75 in 0.1 M NaOAc buffer using *o*-dianisidine as substrate [7] and at 7.0 in 0.1 M KPi buffer, using pyrogallol as substrate [7]. The effect of temperature on the rate of the reaction was studied over the range 20° to 50° under standard assay conditions. A straight line was obtained in the Arrhenius plot with an activation energy of 14 kcal/mol. Table 3 summarizes the principal molecular properties of IP.

Table 2. Amino acid composition of *Ipomoea batatas* seedling peroxidase

Amino acid		% by wt of residues
Aspartic acid	35	13.00
Threonine	27 (a)	8.98
Serine	28 (a)	8.22
Glutamic acid	17	6.99
Proline	15	4.82
Glycine	17	3.56
Alanine	19	4.73
Valine	19	6.21
Methionine	3	1.25
Isoleucine	10	3.66
Leucine	24	8.79
Thyrosine	10	5.06
Phenylalanine	17	7.84
Histidine	5	2.17
Lysine	12	4.89
Arginine	16	7.78
Half-cystine	6 (b)	2.01
Tryptophan	0 (c)	
Total number	280	

Samples containing 0.3 mg of the purified enzyme were hydrolysed for 24, 48 and 72 hr in 1 ml 6 M hydrochloric acid. (a) Obtained by extrapolation to zero hydrolysis time; (b) determined as cysteic acid, and (c) determined by the method of ref. [28].

Table 3. Properties of *Ipomoea* peroxidase

MW (gel filtration)	42 000
MW (SDS-PAGE)	41 000
Fe (g-atoms/mol)	1 (0.11%) (MW 42 600)
Ferriprotoporphyrin	1 (MW 41 300)
Isoelectric point (electrofocusing)	7.3
Carbohydrate	18%
pH optimum (*o*-dianisidine)	5.75
Activation energy (Arrhenius plot)	14 kcal/mole

Amino acid composition

Table 2 reports the amino acid composition of IP obtained by averaging the results from the analyses of the 24, 48 and 72 hr hydrolysates. With the exception of some differences in the quantity of specific amino acid residues, the composition of the protein is similar to those reported for other plant peroxidases [4, 8, 18, 19].

EXPERIMENTAL

Plant material. Tubers of *Ipomoea batatas* were grown hydroponically for 15 days under a controlled environment.

Purification. Fifteen-day-old seedlings (300 g) were homogenized in 1500 ml deionized H_2O for 3 min and the resulting suspension was filtered through two layers of cheese cloth. The suspension was centrifuged at 9000 rpm for 30 min and the ppt discarded (Step 1). The supernatant was made 50% saturated with $(NH_4)_2SO_4$ with constant stirring at 4° over a period of 10 min. The mixture was stirred for an additional 30 min and centrifuged at 9000 rpm for 30 min. The ppt was discarded and the supernatant was made 90% saturated with $(NH_4)_2SO_4$ as before. After centrifugation the pellet was dissolved in 150 ml deionized H_2O. The solution was dialysed against 30 l. H_2O at 4° for 12 hr and the insoluble material removed by centrifugation (Step 2). Although the loss of enzyme activity is very high (*ca* 50%), this step is necessary for purification and we were not able to substitute it with a more useful procedure.

The supernatant was made 0.1 mM with KPi buffer pH 7 and loaded onto a DEAE-cellulose column (2.5 × 10 cm) equilibrated and washed with the same buffer (flow rate 100 ml/hr) until the absorbance at 280 nm was below 0.01. In these conditions the enzyme was not bound to the resin and was collected in the washing (Step 3). The solution from Step 3 was loaded onto a SP-Sephadex column (1 × 5 cm) equilibrated with 0.1 mM KPi buffer pH 7 and eluted with the same buffer until the absorbance at 280 nm was below 0.01. The enzyme was then eluted with 10 mM KPi buffer pH 7 (flow rate 100 ml/hr; 10 ml fractions). The fractions with the highest specific activity were pooled and concentrated by ultradialysis (Step 4). The purification procedure is summarized in Table 1. The overall purification achieved was 10 500 fold with a recovery of about 27%. The purified enzyme was brown in solution like other peroxidases.

Amino acid composition was determined according to ref. [20] with an amino acid analyser LKB model 4101. Standard, single column method was used. Half cystine was determined as cysteic acid after oxidation with performic acid [21]. Serine and threonine contents were extrapolated to zero hydrolysis time. Tryptophan was estimated spectrophotometrically as described in ref. [22].

Other analytical methods. See ref. [10].

Enzyme assay. See ref. [10].

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