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PURIFICATION AND CHARACTERIZATION OF *OPUNTIA* PEROXIDASE

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Key Word Index—*Opuntia ficus indica*; Cactaceae; peroxidase; hydrogen peroxide; plant hemoprotein.

Abstract—Peroxidase from *Opuntia ficus indica* fruits was purified with chromatographic methods. The enzyme had a characteristic spectrum in the visible region and Rz (A_{403}/A_{275}) value of 2.56. It showed a single band in SDS–PAGE electrophoresis. The peroxidase had a M_r of $58\,000 \pm 2000$, an isoelectric point of 7.2 and contained an iron-protoporphyrin IX as prosthetic group. The pH optimum was at 5.75 in 100 mM Na acetate buffer using *o*-dianisidine as substrate. The activation energy was estimated to be 16 kcal mol^{-1} and 50% inactivation occurred after 60 min at 60°.

INTRODUCTION

Peroxidases (EC 1.11.1.7, hydrogen donor oxidoreductase) utilize hydrogen peroxide or substituted peroxides for the oxidation of a large number of substrates. Peroxidases have been found in most plant cells investigated and seem to be a normal component of such cells [1]. 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 the considerable number of isoenzymic species [2]. In tomato plants, Evans and Aldridge [3] separated out six isoperoxidases and in a later paper [4] Evans reported 12 isoperoxidases from tomato shoots. A homogeneous tomato fruit peroxidase isoenzyme was obtained by Jen [5] using hydrophobic chromatography. Isoenzymes were not detected in *Euphorbia characias* peroxidase [6], in *Ipomoea batatas* peroxidase [7] or in *Hordeum vulgare* peroxidase [8]. *Opuntia ficus indica*, widely distributed in dry zones, contains in its fruits high levels of proteins and glucides. Recently in our laboratory we have attempted to purify the *Opuntia* peroxidase and its enzymic, physical and chemical properties have been studied.

RESULTS

Criteria of purity

Opuntia peroxidase obtained by the purification procedure as summarized in Table 1, was tested for homogeneity by PAGE using different conditions. Only one protein band with enzymic activity was present on PAGE or on analytical gel electrofocusing in the pH range 5–8. The isoelectric pH of peroxidase was 7.2. Only one band was observed in SDS–PAGE in the presence and absence of mercaptoethanol. SDS–PAGE showed a peroxidase band with a M_r of $58\,000 \pm 2000$.

Heme content

The heme prosthetic group was identified as iron-protoporphyrin IX (Fe-PPIX) as determined by the oxidized and reduced forms of pyridine hemochromogen derivative. The minimal M_r was calculated to be 55 900 using a molar extinction coefficient of 34.2×10^3 for the 557 nm absorption peak of the pyridine derivative of Fe-PPIX. The average M_r, obtained by the two methods used, yielded a value of 57 000. The enzyme consists of a single polypeptide chain containing one iron atom per mole.

Spectroscopic properties

The spectrum of the oxidized peroxidase shows absorption maxima, in the visible region, at 403, 490 and 640 nm with an ε at 403 nm $= 90.5 \times 10^3$ M^{-1} cm^{-1}. In the UV region, the protein absorption peak is centred at 275 nm with an $\varepsilon = 35.3 \times 10^3$ M^{-1} cm^{-1}. The Rz value (A_{403}/A_{275}) of the purified peroxidase is 2.56.

Substrate specificity

Opuntia peroxidase catalyses the oxidation of many phenols and aromatic amines in the presence of H_2O_2. The most rapidly oxidized substrate is *o*-dianisidine, while 2,2′-azinodi(3-ethylbenzthiazoline-6-sulphonic acid), benzidine, guaiacol and syringaldazine are oxidized with decreasing rates. *Opuntia* peroxidase shows indole acetic acid activity (K_m 6×10^{-5} M) in the presence of dichlorophenol and manganese ions.

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Table 1. Purification of *Opuntia ficus indica* peroxidase

Step	Total protein (mg)	Sp. act. (Units mg^{-1})	Total act. (Units)	Purification (fold)	Recovery (%)
Crude homogenate	390	145	105 000	1	100
Ammonium sulphate fractionation	310	171	98 600	1.2	94
DEAE cellulose column	86	551	87 500	3.8	83
Hydroxylapatite column	2.3	11 900	50 000	82	47

The substrates used to determine the activities are *o*-dianisidine (22 mM) and H_2O_2 (12 mM) in 100 mM NaOAc buffer, pH 5.75.

Effect of pH, buffer and temperature

The effect of pH on peroxidase activity was tested in NaOAc and in KPi buffers. The pH curve showed an optimum at 5.75 in NaOAc buffer using *o*-dianisidine as substrate. The effect of temperature on the rate of the reaction was studied over the range 25–70° under standard assay conditions. A straight line was obtained in the Arrhenius plot with an activation energy of 16 kcal mol^{-1}. On heating at 60°, 50% inactivation occurred after 60 min.

EXPERIMENTAL

Chemicals. Chemicals were obtained as pure commercial products and used without further purification. DEAE cellulose was from Serva; protein standards for SDS–PAGE and hydroxylapatite (Bio Gel HTP) were from BioRad. Protein standards for isoelectrofocusing were from Sigma.

Enzyme assay. Peroxidase 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. The complete reaction mixt. contained: 100 mM NaOAc buffer, pH 5.75, 22 mM *o*-dianisidine, 12 mM H_2O_2 and enzyme in a final vol. of 3 ml. The 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.

Analytical PAGE. Electrophoresis under non-denaturing conditions was performed according to ref. [9]. The isoelectric point was measured by PAGE in the range 3–10 and 5–8 as described in ref. [10]. The standard proteins used were: β-lactoglobulin (5.1), carbonic anhydrase II (5.9), carbonic anhydrase I (6.6), myoglobin (7.2). Continuous SDS–PAGE was carried out according to ref. [11]. The proteins with the following M_r were used as standard for M_r determination: lysozyme (14 400), soybean trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (42 700), BSA (66 000) and phosphorylase B (97 400).

Other analytical methods. Protein was estimated according to ref. [12]. Heme concn was determined according to ref. [6].

Plant material. Mature fruits were from Sardinia's countries.

Purification procedure. Fruits (1 kg) were homogenized in a Waring Blendor with 2 l deionized H_2O for 3 min and the resulting suspension was pressed through a cotton cloth, centrifuged at 14 300 *g* for 30 min and the ppt discarded (Step 1). The supernatant was made 90% satd with $(NH_4)_2SO_4$ with constant stirring at 4° over a period of 30 min and centrifuged at 14 300 *g* for 30 min. After centrifugation the pellet was dissolved in 200 ml deionized H_2O. The soln was dialysed against 30 l deionized H_2O at 4° for 12 hr and the insoluble material removed by centrifugation (Step 2). The supernatant was loaded onto a DEAE cellulose column (2 × 5 cm; Cl^- form) equilibrated and washed with deionized H_2O until the *A* at 280 nm of the effluent became less than 1. In these conditions the enzyme was not bound to the DEAE cellulose. The frs with peroxidase activity were collected (Step 3). The eluate from DEAE was loaded onto a hydroxylapatite column (2 × 5 cm), equilibrated and washed with deionized H_2O until the *A* at 280 nm of the effluent became 0.025. The bound peroxidase was eluted with 100 mM KPi buffer, pH 7. The active frs were pooled and lyophilized (Step 4). The purification procedure is summarized in Table 1. The overall purification achieved was 82-fold with a recovery of *ca* 47%. The purified peroxidase was brown in soln like other peroxidases.

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