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ISOLATION AND CHARACTERIZATION OF ISOPEROXIDASES FROM DEE-GEO-WOO-GEN RICE SEEDLINGS

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Abstract—Multiple forms of peroxidase from ten-day-old Dee-Geo-Woo-Gen (DGWG) rice seedlings were isolated by ammonium sulphate precipitation, polyacrylamide gel electrophoresis (PAGE) and DEAE-cellulose chromatography. The pH optima for P1-A and P1-B are 6.5–7.0 and 7.0 respectively. These isoperoxidases have apparently similar MWs and differ only in their electrophoretic and catalytic properties.

INTRODUCTION

It is known that the peroxidase activity and its isoenzyme patterns alter with changes in the plant development [1]. Peroxidases are known to be actively associated with various functions such as lignin synthesis and cell development [2, 3]. Spontaneous [4] and induced [5] dwarfs exhibit a several-fold increase in specific activity of peroxidase compared to controls. Specific quantitative and qualitative differences for peroxidase isoenzymes were observed in dwarf mutants of TK and IR-8 rice cultivars [6].

Individual peroxidase isoenzymes have been isolated and characterized from a number of plants [7]. These generally differ in MWs, amino acid and sugar composition, kinetic properties and substrate specificity. Different isoenzymes oxidize various substrates, mainly phenolic in nature. Some can also oxidise IAA under certain conditions [8].

The present investigation mainly deals with the isolation and kinetic properties of two isoperoxidases from 10-day-old seedlings of DGWG rice cultivar, since this gene is frequently exploited in breeding programmes for high yielding semi-dwarf varieties.

RESULTS AND DISCUSSION

The results of isolation and purification of peroxidase isoenzymes of P1-A and P1-B are summarized in Table 1. A broad range of ammonium sulphate concentrations (35–90%) were necessary to precipitate fully the solubilized peroxidase activity, presumably because of the presence of multiple enzyme forms. The percentage of total activity recovered in the crude enzyme fraction was 77.1%. Specific activity was increased by 2.55 fold in the crude enzyme fraction. Separation of crude enzyme through polyacrylamide gel electrophoresis resulted in three major isoenzymes. These were designated as P1, P2 and P3 on the basis of their migration. The R_f values of these three anionic peroxidase isoenzymes were found to be 0.1, 0.30 and 0.54 respectively.

The P1-isoperoxidase was further resolved into P1-A and P1-B on a DEAE-cellulose column. There was an increase of seven and 17-fold in specific activity of the purified fraction of P1-B and P1-A respectively. Both of these purified fractions showed only a single protein and enzyme on SDS–PAGE. Gel electrophoresis and ion-exchange chromatographic methods could, thus, yield effective separation of closely associated peroxidase iso-

Table 1. Purification and enzyme activity of P1-A and P1-B fraction of isoenzymes

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Recovery (%)
Homogenate	630	1290	2.05	100
$(NH_4)_2SO_4$ saturation (35–90%) crude peroxidase	190	997	5.3	77.1
After DEAE-Cellulose column				
P1-A	1.5	52.2	34.8	4.0
P1-B	1.0	14.6	14.6	1.12

An aliquot of 30 mg of crude enzyme was applied for preparatory PAGE.

enzymes. Percentage recoveries of P1-A and P1-B from crude protein were up to 4.0 and 1.2 respectively. P1-A moved more slowly than P1-B on SDS–PAGE.

Kinetic studies

The affinity of these two (P1-A and P1-B) purified isoperoxidases was studied with different concentrations of pyrogallol. With increase in the concentrations of pyrogallol as an H-donor, (rectangular hyperbola curves), a typical Michaelis–Menten saturation curve was obtained. These isoenzymes exhibited differences in K_m and V_{max} values. The K_m for pyrogallol of P1-A and P1-B were 8.6 and 5.1 mM and V_{max} 2.84 and 1.08 μmol/min/mg protein respectively. P1-B, thus, shows a higher affinity for pyrogallol than P1-A. The higher affinity of P1-B might play an important role in the oxidation of phenolic compounds probably as a defence mechanism. The effect of pH ranging from 5 to 9 on isoperoxidase activity was studied. The pH optima for P1-A and P1-B were slightly different for P1-A activity; the optimal pH was 6.5–7.0, and for P1-B it was 7.0. Such observations were also cited for tobacco leaves [9] with two isoenzymes having pH optima 7.0 and 7.5, and in peanut [10] four isoenzymes having optima at pH 6–8.

MWs

SDS–PAGE showed only one protein band in P1-A and P1-B. When incubated with or without mercaptoethanol before electrophoresis no dissociation was observed in the gel patterns. The isoenzymes had similar mobility with an apparent MW of 60000.

A wide range in MW has been reported in the literature for peroxidase isoenzymes from various sources. These include values of 60000 for four peanut isoperoxidases [11]; 38700, 50000, 51000, 51600 for four turnip isoperoxidases [12]; 33400, 45000, 57000 for three ribosome-associated isoperoxidases from lentil roots [13]; 40000 and 50000 for red alga isoperoxidases [14]; 30000, 40000, 44000 and 54500 for Japanese radish isoperoxidases [15, 16] and 40000 for five isoperoxidases from horseradish [17]. Thus the MW of peroxidases ranged between 30000 to 60000 and the isoenzymes studied in the present investigation had relatively higher MW.

EXPERIMENTAL

The experimental procedures described below were all carried out at 4°.

Isolation of peroxidases. 10-day-old DGWG seedlings of rice were washed with H_2O, cut into pieces and homogenized with 0.1 M K_2HPO_4 in a Waring blender, the resulting homogenate was then squeezed through eight layers of cheese cloth.

Ammonium sulphate fractions. The filtrate was brought to 35% satn with $(NH_4)_2SO_4$ and centrifuged at 10000 rpm for 15 min. The pellet was discarded and the supernatant was brought to 90% satn with $(NH_4)_2SO_4$. After standing for 18 hr, the soln was centrifuged at 10000 rpm for 15 min. The pellet was dissolved in 5 mM Tris–HCl buffer (pH 7) and then dialysed against 5 mM Tris–HCl (pH 8) containing 0.1 M KCl for 48 hr. The dialysate was centrifuged at 10000 rpm for 15 min and the supernatant was lyophilized and designated as crude peroxidase fraction.

PAGE. The crude peroxidase was separated on a slab gel into three isoperoxidases with P1 (A and B) having slow mobility and P2 and P3 with fast mobility. Samples in 40% sucrose soln were applied on 7.5% polyacrylamide gel slabs of 1.5 mm thickness. Tris–glycine (pH 8.3) buffer was used in both reservoirs and electrophoresis was run for 3 hr at a constant current of 3 mA per column at 4°. At the end of the electrophoresis, the gel was cut into two pieces, one of which was stained for peroxidase activity and the other for protein. The remaining gels were cut into three portions (P1, P2 and P3 isoperoxidases) with the stained pieces as reference. The individual fractions were then homogenized in a Waring blender at maximum speed for 5 min. The supernatant fractions were lyophilized and designated as partially purified fractions of P1 (A and B), P2 and P3.

Ion-exchange chromatography. The partially purified isoenzyme (P1), after dialysis against 5 mM Tris–HCl buffer pH 7.5 was separated on a DEAE-cellulose column (18 × 0.8 cm) previously equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl soln made up of 500 ml of Tris–HCl buffer (5 mM, pH 7.5) and 500 ml 0.2 M NaCl in Tris–HCl buffer. Fractions of 5 ml each were collected and analysed for protein and enzyme activity (Fig. 1). The purity of

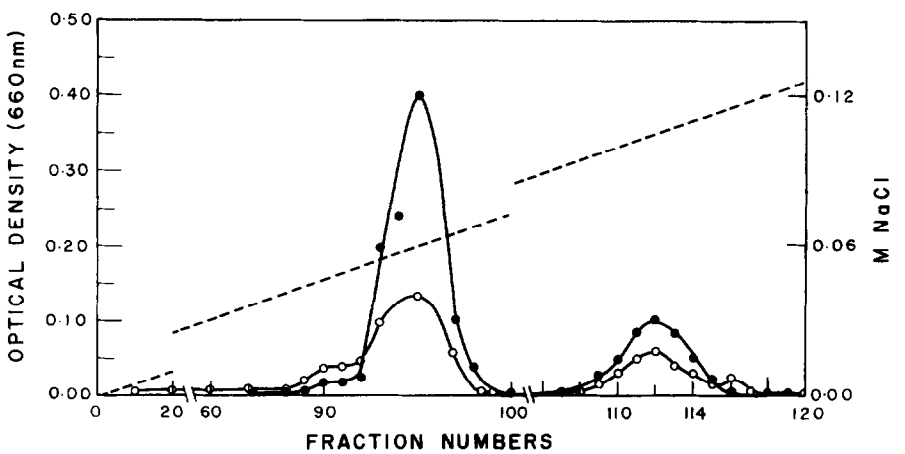

Fig. 1. Separation of P1 on DEAE-cellulose column. The flow rate was 20 ml/hr, the fraction volume 5 ml, and 20 μl aliquots were taken for analysis for both protein estimation and enzyme activity. (●●●) *A* at 420 nm; (○○○) *A* at 660 nm; (– – –) NaCl concentration.

the isoenzymes of P1-A and P1-B was checked again by electrophoresis.

Enzyme assay. This was carried out according to the procedure of ref. [18]. The assay system contained 125 μmol of 0.1 M NaPi buffer (pH 6.8), 50 μmol of pyrogallol and 50 μmol of H_2O_2 and 1 ml of enzyme soln in 5 ml total vol. The reaction was terminated by adding 0.5 ml of 5% H_2SO_4 after incubating at 20° for 5 min. The amount of purpurogallin formed was determined by using the extinction coefficient $C_{420} = 3.162$/cm/min and the enzyme activity was expressed in terms of μmol of product/min/mg protein. Protein was determined by the method of ref. [19].

Staining. Gels were stained with 0.25% Coomassie brilliant blue in a solution of HOAc–MeOH–H_2O (2:3:15) for 1 hr to locate the protein bands, and destained with the same soln without the dye. Isoperoxidases were stained with a soln consisting of benzidine; NH_4Cl (30%); H_2O_2 (25:5:6). The gels were incubated for 10 min at room temp., destained in a soln of 7% HOAc and fixed in 2% HOAc. The pattern of isoperoxidases on polyacrylamide gel in crude extract and after ion-exchange resolution, was scanned on a Shimadzy UV-VIS Double Beam 240 Spectrophotometer.

MW determination. MWs of the P1-A and P1-B isoperoxidases were determined by SDS–PAGE using albumin, ovalbumin and lysozyme as calibration markers [20].

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