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KINETIC PROPERTIES OF IAA OXIDASE FROM MUNG BEAN COTYLEDONS

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Key Word Index—*Vigna radiata*; Leguminosae; mung bean; cotyledons; peroxidase; IAA oxidase; kinetic properties.

Abstract—A crude enzyme preparation from mung bean cotyledons was separated into peroxidative and non-peroxidative IAA oxidase on a DEAE–cellulose column. Both fractions differed in their pH optima, K_m and V_{max}. The K_m and V_{max} of non-peroxidative IAA oxidase were higher than those of peroxidative IAA oxidase. Peroxidative IAA oxidase showed a linear increase in absorption at 247 and 254 nm after a short lag of 2–3 min. The addition of catalytic amounts of hydrogen peroxide eliminated the lag period and also enhanced the rate of IAA degradation. The non-peroxidative IAA oxidase fraction, however, did not exhibit any significant increase in absorption at 247 and 254 nm and showed a lag period of 5 min which was not affected by hydrogen peroxide. Instead, addition of the same catalytic amount of hydrogen peroxide inhibited the rate of IAA degradation. The peroxidative IAA oxidase fraction exhibited the reaction kinetics characteristic of peroxidase-catalysed IAA degradation. The rate of IAA oxidation by purified non-peroxidative IAA oxidase was very low. The slow rate of catalysis shown by non-peroxidative IAA oxidase appears to be due to the presence of inhibitor(s).

INTRODUCTION

IAA oxidase and peroxidase activities in plant cells are generally known to occur in the same protein [1]. This view is based on the observations that many IAA oxidase preparations contain peroxidase and IAA oxidase activity is affected by the factors which affect peroxidase activity [2–6]. Attempts to separate the two activities from some plant tissues have been unsuccessful. Both types of activities move together during purification [3–5, 7]. Sequeira and Mineo [8], however, claimed to have separated the peroxidase and IAA oxidase activities from tobacco roots on an SE–Sephadex column. Non-peroxidative IAA oxidase has also been isolated from crown gall tissue culture cells [9]. In the present investigation IAA oxidase has been partially separated from peroxidase from mung bean cotyledons and its kinetic behaviour has been studied.

RESULTS AND DISCUSSION

IAA oxidase and peroxidase activities in germinating cotyledons

IAA oxidase and peroxidase activities were determined in de-embryonated mung been cotyledons, incubated at $28 \pm 2°$ for various time intervals. There was no detectable peroxidase activity for 4–6 hr and no IAA oxidase activity for 24 hr after incubation. Thereafter, the activities of the two enzymes increased gradually until 96 hr after incubation. Crude extracts of 48 and 72 hr old cotyledons revealed two (R_f 0.82 and 0.63) and three isoenzymes (R_f 0.82, 0.63 and 0.48) of IAA oxidase, respectively, on polyacrylamide gels.

Partial separation of IAA oxidase from peroxidase

The specific activity of IAA oxidase in crude homogenates of 72 hr old cotyledons was 10.34 units/mg protein. During fractionation of crude extracts on a DEAE–cellulose column two major IAA oxidase containing activity peaks were obtained; the first peak contained peroxidase activity, whereas the second peak contained only negligible peroxidase activity. Peroxidative IAA oxidase was purified four-fold and showed a slow moving isoenzyme (R_f 0.82) in polyacrylamide gels, while the non-peroxidative IAA oxidase fraction was purified 25-fold and contained two IAA oxidase isoenzymes (R_f 0.82 and 0.67). Another peroxidase activity peak devoid of IAA oxidase activity was also obtained after elution of the column with a sodium chloride gradient (Fig. 1).

Sequeira and Mineo [8] have isolated a non-peroxidative IAA oxidase from tobacco roots and a similar enzyme from commercial horseradish peroxidase. Bryant and Lane [10] have electrophoretically separated IAA oxidase and IAA peroxidase from various parts of pea seedlings and found that IAA oxidase isoenzymes of plumule hooks were devoid of peroxidase activity. The non-peroxidative IAA oxidase fraction from mung bean cotyledons, or its isoenzyme on polyacrylamide gels, did not contain any detectable peroxidase activity.

Kinetic properties of peroxidative and non-peroxidative IAA oxidases

Peroxidative and non-peroxidative IAA oxidase fractions showed pH optima of 6.0 and 6.9, respectively. The IAA oxidase of mung bean cotyledons was thermostable. A sharp increase in IAA oxidase activity in crude extracts after 1–2 min at 60° suggested the possible presence of a

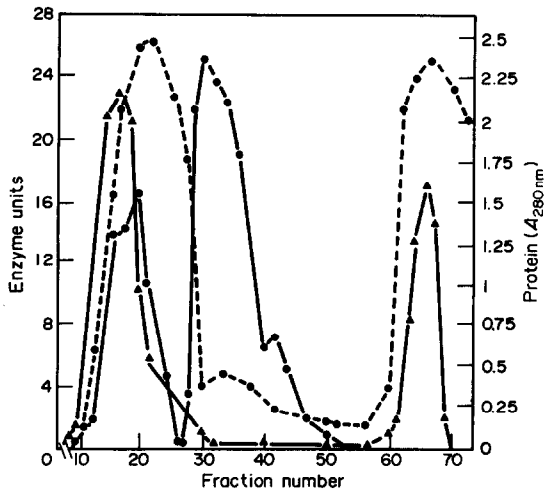

Fig. 1. DEAE-cellulose elution profile of peroxidase and IAA oxidase. Cell-free extracts of mung bean cotyledons, incubated at 28 ± 2° for 72 hr in water, were prepared and then fractionated on a DEAE-cellulose column as described in the Experimental. IAA oxidase activity was assayed using Salkowski reagent. IAA oxidase activity (●—●); peroxidase activity (▲—▲); protein (●----●).

thermolabile inhibitor(s) of the enzyme. A similar rise in the activity of non-peroxidative IAA oxidase after 5 min indicated that the inhibitor(s) was eluted along with the non-peroxidative IAA oxidase fraction during purification (Fig. 2). The K_m and V_{max} values for non-peroxidative IAA oxidase (0.71 mM and 244 units/mg protein, respectively) were higher than those for peroxidative IAA oxidase (0.38 mM and 56 units/mg protein, respectively).

The oxidation products of IAA are known to absorb strongly at 247 and 254 nm and, therefore, the increase in A at 247 and 254 nm was taken as a measure of the rate of IAA oxidation. The peroxidative IAA oxidase fraction showed a linear increase in A at 247 and 254 nm after a lag of 2–3 min, whereas the non-peroxidative fraction exhibited a slight but linear increase after a longer lag period of 5 min (Fig. 3). It is evident that oxidation of IAA results in a number of unstable intermediates leading to the formation of a product with A maxima at 247 and 254 nm.

Peroxidative IAA oxidase from mung bean exhibited the reaction kinetics characteristic of peroxidase-catalysed IAA oxidation, as reported by Ray [11], and Hinman and Lang [12]. The reaction kinetics exhibited by non-peroxidative IAA oxidase were similar to those shown by IAA oxidase from other sources, such as *Omphalia flavida* [11, 13], tobacco roots and commercial horseradish peroxidase [8].

Addition of catalytic amounts of hydrogen peroxide to the assay mixture of peroxidative IAA oxidase produced a rapid increase in A at 247 nm, eliminating the short lag period (Fig. 4). Peroxidase-catalysed IAA oxidation is known to be enhanced by hydrogen peroxide. A similar effect of hydrogen peroxide on IAA oxidation by peroxidase containing IAA oxidase has been reported from tobacco roots [8]. Ray [13] and Manicol [14] have also reported that small amounts of hydrogen peroxide reduce or eliminate the lag period of the IAA oxidation reaction catalysed by plant peroxidases. Kokkinakis and Brooks [15] reported that hydrogen peroxide was required not only to initiate the reaction by tomato peroxidase but also

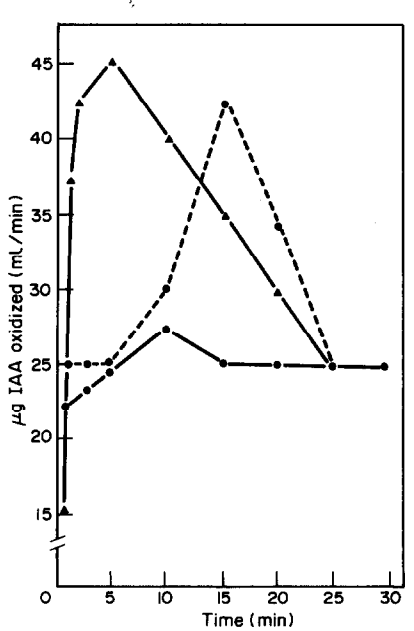

Fig. 2. Effect of temperature on IAA oxidase activity in crude extract, and peroxidative and non-peroxidative fractions. Experimental details as in Fig. 1 except that the cotyledons were incubated in 5×10^{-4} M IAA. The various fractions were incubated at 60° for various periods as indicated. Crude extract (▲—▲); peroxidative (●—●); non-peroxidative (●------●).

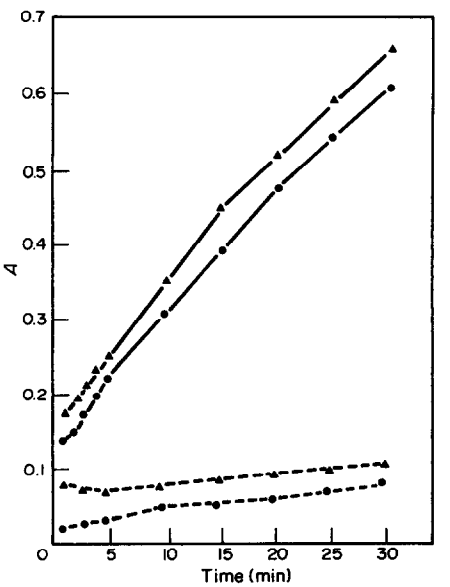

Fig. 3. Kinetics of IAA oxidation by peroxidative and non-peroxidative fractions. Fractions were collected as described in the Experimental. The change in A was recorded at 247 nm (●) and 254 nm (▲). Peroxidative (—); non-peroxidative (----).

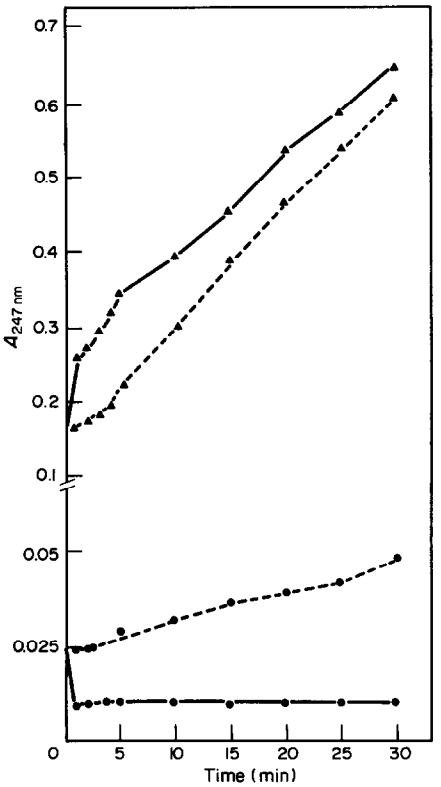

Fig. 4. Effect of hydrogen peroxide (3 μmol/ml) on the kinetics of IAA oxidation by peroxidative (▲) and non-peroxidative (●) fractions. Reaction mixtures as indicated in the Experimental, except for the addition of 0.02 ml 0.4 M hydrogen peroxide at zero time. No hydrogen peroxide (- - - - -); with hydrogen peroxide (——).

to maintain the supply of intermediates. However, addition of the same catalytic amount of hydrogen peroxide to the assay mixture of non-peroxidative IAA oxidase resulted in inhibition of IAA degradation (Fig. 4). IAA oxidase from tobacco roots was also inactivated by hydrogen peroxide, although at very low concentrations it slightly increased IAA oxidase activity [8]. The inhibition by hydrogen peroxide could result either from its non-specific attack on S–H bonds or from the formation of complex III, as suggested for tobacco IAA oxidase [8].

The higher K_m shown by non-peroxidative IAA oxidase clearly indicates that this fraction possessed less affinity towards IAA than that of peroxidative IAA oxidase, although the former was a more purified preparation of IAA oxidase than the latter. Preincubation of the enzyme preparations at 60° indicated the presence of a thermolabile inhibitor(s) in mung bean cotyledons which apparently coelute(s) with the non-peroxidative IAA oxidase fraction during purification. The lower A by non-peroxidative IAA oxidase products at 247 and 254 nm and its low affinity towards IAA may be attributed to the presence of inhibitor(s).

EXPERIMENTAL

Germination of cotyledons. Seeds of mung bean (*Vigna radiata* L. wilczek var. Pusa Baisakhi) were surface sterilized in 0.1% $HgCl_2$ for 5 min and washed thoroughly with sterile H_2O. The seeds were soaked in sterile H_2O for 6–8 hr at 16°. The seed coat and embryonic axis were removed and, after thorough washing, the de-embryonated cotyledons were spread over acid-washed quartz sand placed in Petri dishes under aseptic conditions. The quartz was moistened with sterile H_2O containing chloramphenicol (50 μg/ml). The Petri dishes containing cotyledons were kept for various periods in an incubator at 28 ± 2°.

Enzyme extraction. After the desired incubation time, the cotyledons were washed with sterile H_2O, blotted dry and homogenized in 0.02 M Tris–HCl buffer, pH 7.6, with a pestle and mortar, using white sand as an abrasive. The homogenate was centrifuged at 10000 *g* for 30 min. The supernatant fraction (crude extract) was employed for measuring IAA oxidase and peroxidase activities. All the operations were carried out at 4°.

DEAE–cellulose column chromatography. A 25 × 2 cm column was packed with thoroughly washed DEAE–cellulose and equilibrated with 0.02 M Tris–HCl buffer (pH 7.6). The crude enzyme extract (20 ml or 174 mg protein) was added gently to the column and fractions of 5 ml/3 min collected with an automatic fraction collector. The non-exchanged protein was eluted with 350 ml 0.02 M Tris–HCl buffer (pH 7.6) and fractions collected. The exchanged protein was eluted by a continuous ionic gradient from 0 to 1 M NaCl. The column was run at 4–6°.

Assay of peroxidase activity. Peroxidase was assayed by the method of Ref. [16]. The assay mixture comprised *o*-dianisidine (2.4 μmol), H_2O_2 (20 μmol), crude extract (0.05–0.5 mg protein) and 0.05 M citrate buffer (pH 4.8) to make the vol. up to 4 ml. Omission of H_2O_2 from the incubation mixture served as a blank. The enzyme activity was measured by following the A at 430 nm at intervals of 15 sec. One unit of enzyme activity represents a change of 1 A unit/min at 430 nm in an incubation mixture of 4 ml.

Assay of IAA oxidase activity. IAA oxidase activity was determined both by colorimetric and spectrophotometric methods. A modified procedure of ref. [17] was used for the colorimetric determination of IAA oxidase activity. The assay mixture contained 0.2 ml IAA (20 μg), 0.2 ml enzyme extract, 0.05 ml 2,4-dichlorophenol (0.05 mM), 0.05 ml $MnCl_2$ (0.05 mM) and 0.1 M citrate buffer (pH 6) in a final vol. of 1 ml. The mixture was incubated in the dark at 30° for 15 min. Salkowski reagent (2 ml) was added to each tube and the assay mixture incubated at 30° for 30 min. Salkowski reagent was prepared by adding 1 ml freshly prepared $FeCl_3$ (8% w/v) to 50 ml 35% $HClO_4$. The A of the colour developed was measured at 530 nm. Omission of IAA from the assay mixture served as a blank. The assay mixture without enzyme extract served as an IAA standard. One unit of enzyme activity represents 1 μg IAA hydrolysed/ml · 10 min.

The procedure of ref. [8] was followed for the spectrophotometric determination of IAA oxidase activity. The reaction mixture contained 0.125 ml 1×10^{-3} M 2,4-dichlorophenol, 0.375 ml of a mixture of 1×10^{-3} M IAA and 5×10^{-4} M $MnCl_2$, 2.0 ml 0.2 M KH_2PO_4, pH 4.7, and 0.25 ml enzyme extract. The substrate, cofactors, buffer and enzyme extract were incubated separately at 34° before mixing in a cuvette. The enzyme extract was added to the sample cuvette at zero time and the change in A recorded at 247 and 254 nm.

Estimation of protein. Protein was estimated according to ref. [18] using crystalline BSA as a standard.

Separation of IAA oxidase isoenzymes on polyacrylamide gels. IAA oxidase isoenzymes were separated on polyacrylamide gels in an anionic system according to refs [19, 20]. The IAA oxidase isoenzymes were developed by the modified procedure of Ref. [5]. The gels were immersed in a staining mixture containing 1 part of soln A (4 mg Fast Blue BB/ml EtOH), 2 parts of soln B (16.4 mg *p*-coumaric acid + 17.6 mg IAA dissolved in EtOH to a

total vol. of 50 ml + 2.4 ml 0.1 % H_2O_2) and 1 part of soln C(2 M acetate buffer, pH 4.2), until the brown bands of IAA oxidase appeared.

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