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Peroxidase and IAA Oxidase in Germinating Seeds of *Cicer arietinum* L.

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Peroxidase and indole acetic oxidase (IAA oxidase) activities in seeds of *Cicer arietinum* L. were studied at 72 h of germination. Both activities behaved differently in relation to optimum pH, stability against temperature and time of storage at 4° C.

Disc electrophoresis on polyacrilamide gel showed the existence of five anionic and three cationic isoenzymes with peroxidase activity by using benzidine as substrate; three of the anionic isoenzymes and one cationic isoenzyme possessed a simultaneous IAA oxidase activity as well.

Indole acetic acid (IAA) is the best known naturally occurring auxin, and it participates in controlling many phases of growht and differentiation. It is a well established fact that plants contain enzymes capable of oxidizing IAA *in vitro* (15). Although there are some exceptions (16, 21) it seems that these enzymes also possess peroxidase (EC 1.11.1.7) activity (4, 7). Several isoenzymes with peroxidase activity are found in plants (17, 19) performing a wide variety of functions, but there are conflicting results as to the ability of these isoenzymes to oxidize IAA, indicating that the isoenzymic relationship of IAA oxidase to peroxidase might vary from species to species.

Four hypotheses have been put forward in order to explain the relationship between IAA and peroxidase: *a)* Both activities are different enzymes (16). *b)* There exist several peroxidase isoenzymes in which only some posses IAA oxidase activity (20). *c)* Peroxidase and IAA oxidase activities are associated with the same protein molecules but they have different active sites or involve differential allosteric activation (2, 13, 19). *d)* Peroxidase and IAA oxidase are identical, IAA is merely another substrate (7, 18).

The scope of this report is to study the characteristics of both catalytic activities in *Cicer arietinum* L. seedlings in order to find out whether isoenzymes catalizing oxidation of IAA are separable from peroxidase.

Materials and Methods

Extraction of crude enzyme. Seeds of *Cicer arietinum* L. were sterilized for 3 min with 1 % calcium hypochlorite rinsed

several times with sterile distilled water and soaked in sterile distilled water for 3 h and then grown in the dark on wet filter paper in Petri dishes —7 seeds and 7 ml of sterile distilled water per dish— for 72 h at 25 ± 1° C. The crude enzyme was prepared by blender homogenizing 30 g of seedlings plus 15 g of polyvinyl pyrrolidone (Polyclar AT) with 150 ml of cold 0.05 M phosphate buffer (pH 5.8). The homogenate was filtered through a triple layer of cheesecloth and the residue was washed twice with 50 ml of 0.05 M phosphate buffer (pH 5.8). The filtrates were combined and centrifuged at 20,000 *g* for 15 min. To the supernatant cold acetone was added and it was then centrifuged again at 20,000 *g* for 15 min. The pellet was resuspended in 100 ml of 0.05 M phosphate buffer (pH 5.8), cold acetone was added again and after centrifugation the pellet was resuspended in 50 ml of 0.05 M phosphate buffer (pH 5.8). The enzyme solution was dialyzed against 4 l of 5 mM buffer phosphate (pH 5.8) for 24 h. All the steps of the above procedure took place at 4° C. The enzyme extract was used immediately but sometimes it was neccessary to store it and this was done by freezing at —15° C or lyophilized until required for further analysis, with no loss of activity up to 6 months.

IAA oxidase assay. An adaptation of already described methods (9, 11, 12) was used to assay IAA oxidase, measuring residual IAA, following dark incubation with shaking at 30° C. The enzyme incubation mixture contained: 1 ml 0.005 M phosphate buffer (pH 5.8), 0.5 ml 5×10^{-4} $MnCl_2$, 1 ml 10^{-3} M IAA and 2 ml of crude enzyme giving a total volume of 5 ml. Reaction was initiated by the addition of IAA. Residual IAA was determined colorimetrically. Aliquots of 1 ml were withdrawn from the incubation mixtures after 0 and 60 min incubation periods and placed in flasks containing 4 ml of the Salkowski reagent. After incubating in the dark for 60 min, absorbance was measured at 530 nm. One IAA oxidase unit was defined as the amount which degrades 1 μg of IAA $\times$ h^{-1}. A blank was set up with heat denaturated (5 min at 50° C) enzyme.

Peroxidase assay. The assay of peroxidase activity was a variation of the VAN LELYVELD and PRETORIUS method (22). The enzyme incubation mixture contained: 0.5 ml 0.05 M citrate buffer (pH 4.6), 0.5 ml 4×10^{-4} guaiacol, 1 ml 0.3 % H_2O_2 and 0.5 ml enzyme preparation, giving a total volume of 2.5 ml. The reaction was started by the addition of the enzyme. The mixture was incubated for 5 min at 25° C. The changes in the OD were measured at 420 nm. A blank was set up with a heat denaturated (5 min at 50° C) enzyme. One unit of enzyme activity was defined as the amount which produced a change in absorbance of 0.01 $\times$ min^{-1}.

Protein concentration was determined by LOWRY's method (8) using bovin serum albumin as standard.

Gel filtration column chromatography. The enzyme extract was analyzed by gel filtration on a Sephadex G-100 column (2.5 $\times$ 30 cm), equilibrated with 0.05 M phosphate buffer (pH 5.8). The absorbance of the effluent at 254 nm was recorded and IAA oxidase or peroxidase activity of the fractions was determined as above.

Electrophoresis. The polyacrylamide disc electrophoresis for anionic (3) and cationic (14) separations was carried out as described. The staining mixture for IAA oxidase contained 1 mM IAA, 0.1 mM 2,4-dichlorophenol and 0.5 mM $MnCl_2$ in 0.1 M phosphate buffer, pH 5.5, and then p-N,N-dimethylaminocinamaldehyde (5) or blue rapid (4) was added. Peroxidase was detected by incubating the

gel with three different hydrogen donors: bencidine, o-dianisidine and guaiacol (6).

Results

In the standard assay of IAA oxidase, maximal activity was at pH 5.8 and half maximal activity circa pH 3.8 and pH 7. For peroxidase, maximal activity was between pH 4.5 and 5.5 above pH 6.5. The enzyme is completely inactive.

The effect of temperature on the stability og both activities was studied in the standard reactions. IAA oxidase at pH 5.8 comes down gradually over 10° C and 60° C there is no activity. Peroxidase activity at pH 4.6 is maximal up to 30° C. Afterwards it falls sharply to a point where 17 % of the maximal activity is at 40° C (fig. 1). With regard to the storage temperature the enzyme extract only retained full activity up to 6 months if frozen at —15° C or when lyophilized and kept in the fridge.

Using 2.5×10^{-4} M IAA in the reaction mixture at pH 5.8 the optimal concentration for Mn^{2+} ions of those assayed is 10^{-3} M, and for the phenolic cofactor, resorcinol, 10^{-3} M (fig. 2).

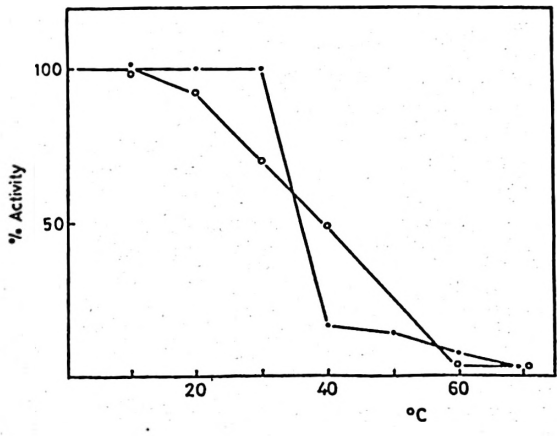

Fig. 1. *Effect of temperature on the stability of IAA oxidase and peroxidase.*
Activity is represented as percentage of the original activity remaining after incubation for 10 min at the indicated temperatures. O—O IAA oxidase, ●—● peroxidase.

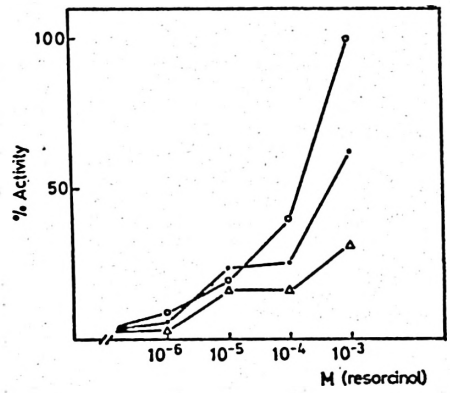

Fig. 2. *Effect of the cofactor resorcinol on IAA oxidase activity.*
In the standard reaction, resorcinol was added at the concentration: 10^{-5} M (Δ—Δ), 10^{-4} M (●—●) and 10^{-3} M (O—O).

If the reaction proceeds under optimal conditions, H_2O_2 has no effect on its rate even at 10^{-2} M.

While peroxidase follows Michaelis Menten kinetics with a guaiacol $K_m = 36 \times 10^{-3}$ M, high concentrations of IAA inhibit the IAA oxidase reaction and at low substrate concentration there is more activity at low protein content (fig. 3).

Gel filtration column chromatography. The enzyme extract analyzed by gel filtration on Sephadex G-100 (fig. 4) shows that the molecular weights of these enzymes are distinctly below the limit of the gel exclusion (150,000 daltons). However, the two systems are found in the same fractions.

Disc electrophoresis. When guaiacol was used to stain for peroxidase isozymes on gels, two anionic and two cationic isozymes were detected (fig. 5). When staining with o-dianisidine, four anionic and three cationic isozymes were detected and if the stain was carried out with

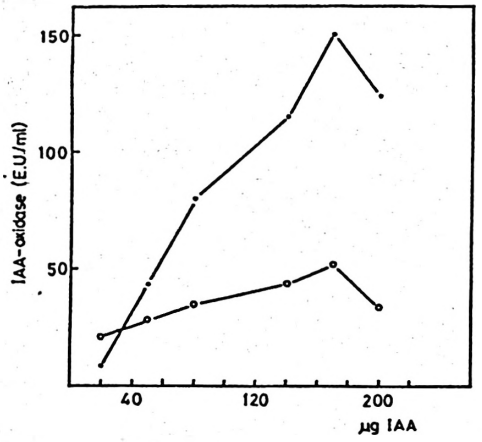

Fig. 3. *Effect of substrate on IAA oxidase activity.*
The standard reaction was carried out with increasing amounts of substrate and at two levels of protein: 500 µg (O—O) and 1,500 µg (●—●).

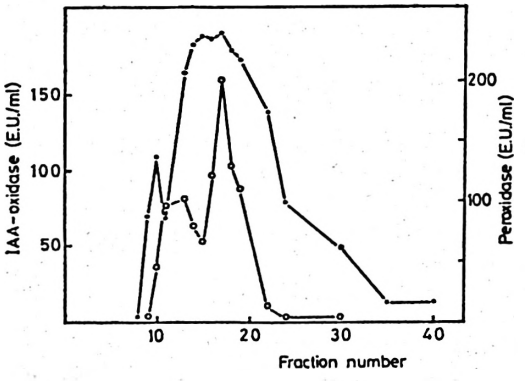

Fig. 4. *Sephadex G 100 filtration.*
IAA oxidase (O—O) and peroxidase (●—●).

bencidine, five anionic and three cationic isoenzymes were detected. Three anionic and one cationic IAA oxidase isoenzymes were also detected and, as can be seen, the electrophoretic mobilities of IAA oxidase isoenzymes were identical with some of the peroxidase isoenzymes.

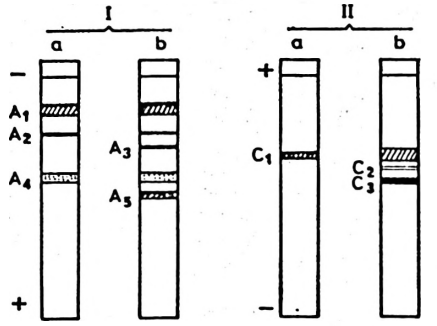

Fig. 5. *Polyacrylamide gel disc electrophoresis.*
Electrophoregrams of IAA oxidase (*a*) and peroxidase (*b*). (I) anionic pH 8.3 (II) cationic pH 4.5.

Discussion

From the above results, some differences between the properties of both enzyme activities in relation to pH, temperature of reaction, stability and kinetics are clear. IAA oxidase kinetics can be justified, if the enzyme has two binding sites (10): a primary one with high affinity for IAA and low catalytic activity and the secondary one with low affinity for IAA and high catalytic activity which starts to operate when the primary site is saturated. This model satisfies our results of low enzyme concentration, which oxidizes more IAA than high enzyme concentration at low substrate level.

IAA oxidase and peroxidase have quite similar molecular weights, but it is clear that Sephadex G-100 filtration cannot give enough evidence to show that the analyzed systems are different or involve the same enzymes.

The isoenzymes separated by electrophoresis from gram seeds may be grouped into two categories: *a*) those having both IAA oxidase and peroxidase activities; *b*) peroxidase with no IAA oxidase activity.

Our results are consistent with other

reports (20): i.e. IAA oxidase is a peroxidase, however that peroxidase is an IAA oxidase has not been proved by our results (6, 7). It has been found in peas (1) and tobacco (16) that some proteins exhibit IAA oxidase activity although some of these results have been criticized on the grounds that IAA oxidase is an artifact resulting from accidental removal of heme during extraction. In the case of gram seeds there is no evidence of such IAA oxidase activity separated from peroxidase.

The reason for different staining of peroxidase isozymes among different hydrogen donors is not known. It may be due to differing degrees of sensitivity between hydrogen donors and certain isozymes which are not activated in the case of some hydrogen donors. As expected, the best results were achieved with bencidine both in clarity and number of bands revealed.

Resumen

Se estudia la actividad peroxidasa y ácido indol acético oxidasa (AIA oxidasa) en semillas de *Cicer arietinum* L. tras 72 h de germinación. El comportamiento de la actividad peroxidasa y AIA oxidasa difiere en pH óptimo, estabilidad frente a temperatura de incubación y almacenamiento a 4° C.

La electroforesis en gel de poliacrilamida pone de manifiesto la existencia de cinco isoenzimas aniónicos y tres catiónicos con actividad peroxidasa, utilizando bencidina como substrato de la reacción, de los cuales tres isoenzimas aniónicos y un isoenzima catiónico poseen a la vez actividad AIA oxidasa.

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