Phytochemistry, 1971, Vol. 10, pp. 2355 to 2359. Pergamon Press. Printed in England.

LOCALIZATION OF ISOZYME FORMS OF PEROXIDASE IN THE COTTON PLANT

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(Received 6 November 1970)

Abstract-Peroxidase activity was assayed in five organs of the cotton plant. Total activity was in the order: leaf > ovary wall (boll) > petiole > stem = root. Peroxidase isozymes were separated from extracts 01 the young leaf, mature leaf, stem, and boll, using acrylamide gel disc electrophoresis. Five isozymes were found in the cotton plant, a major one occurring in each of the organs examined. One isozyme was present only in the boll, and another only in leaves; two isozymes, not present in the young leaf, were present in the old leaf. Subcellular fractionation by differential centrifugation suggested that the cotton leafperoxidase occurs primarily in the cytosol.

INTRODUCTION

PEROXIDASE activity has been found in all higher plants investigated.[1] There is some evidence that peroxidase may be involved in lignification,[2] growth regulation,[3] and disease resistance.[4] Multiple forms of the enzyme occur, and the amounts and patterns of distribution vary with the environment,[5] as a result of injury,[6] and among genetic variants.[7-9]

Altschul et *al.*[10] measured the changes in peroxidase activity in the germinating cotton seedling. Morgan and Hall[11] investigated the changes in peroxidase activity in the cotton seedling shoot and in the leaves of the flowering plants following fumigation with ethylene, Fowler and Morgan[12] studied changes in the peroxidase isozyme pattern in the seedling occurring after ethylene treatment. Otherwise, little has been reported concerning the occurrence, distribution or function of the enzyme in that important agricultural species. It was the object of the present investigation to determine the distribution of peroxidase in the cotton plant (*Gossypium hirsutum* L. var. Auburn 57).

RESULTS AND DISCUSSION

Peroxidases are capable of oxidizing a variety of hydrogen donors; for example, phenolics, aromatic amines, heterocyclics such as indole-3-acetic acid, and inorganic ions

* Supported in part by Memphis State University Summer Research Grant.

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such as iodine. An enzyme having such versatility may have several metabolic functions in the plant. These functions may vary with the tissue or with the subcellular organelle. Horseradish root peroxidase occurs in seven isozyme forms that differ not only in electrophoretic mobility but in their catalytic properties. [9]

The initial rate of oxidation of guaiacol appeared to be first order with respect to cotton peroxidase under the experimental conditions. The rate was proportional to the enzyme concentration over a 100-fold concentration range. The enzyme was rather insensitive to differences in the extraction medium; and was also rather thermostable, retaining 70 % of its original activity after 30 min at 70". The relative peroxidase activity of the cotton plant tissues is shown in Table 1. The activity (18·7 G.U./g fr. wt. of tissue) was highest in the leaf. This was 30–40% of the peroxidase activity found in the horseradish root, on the same fr. wt. basis.

TABLE 1. RELATIVE PEROXIDASE ACTIVITY OF VARIOUS TISSUES OF THE COTTON PLANT

Tissue	Relative activity*
Leaf	100
Petiole	13
Stem	4
Root	4
Boll	33

* The relative activity is $\frac{\text{tissue activity/wt.}}{\text{leaf activity/wt.}}$ x 100.

The peroxidase activity of the various fractions obtained from a typical homogenate of cotton leaf blade tissue is shown in Table 2. Ninety-two per cent of the peroxidase and 42 % of the protein remained in the 100,000 g supernate, which constituted 90 % of the volume of the whole homogenate. The cotton leaf peroxidase thus appears to be a soluble enzyme. Similar experiments using soybean leaves and Japanese radish leaves and roots indicated

TABLE 2. DISTRIBUTION OF PEROXIDASE ACTIVITY IN SUBCELLULAR FRACTIONS OF COTTON LEAF TISSUE

	Fraction*	Total peroxidase activity G.U.†	Total protein mg	Specific peroxidase activity G.U./mg protein
I	Whole homogenate	355·0	965	0·37
II	180 *g* pellet	3·8	57	0·066
III	6000 *g* pellet	11.4	331	0.034
IV	35,000 *g* pellet	4.4	1.57	0.027
V	100,000 *g* pellet	1·3	40	0·033
VI	Supernate	328.0	405	0.81
	Recovery (%)	98.6	102.5	—

* See text for methods employed.
† Guaiacol Units as defined in text.

that their peroxidase is also predominantly soluble. Tolbert et *al.*[13] found 'some' peroxidase activity in particulate fractions of spinach leaves, though the 'bulk' was in the supernate. They were able to detect no activity in peroxisome fractions that were isolated by sucrose density centrifugation. Plesnicar et *al.*[14] reported that over 90 % of the peroxidase activity in homogenates of etiolated mung bean hypocotyls was soluble; but they concluded that much of the enzyme was originally localized in microbodies or lysozomes which were ruptured by tissue homogenization. Hackett and Ragland[15] reported finding some peroxidase activity in various subcellular fractions they obtained by differential centrifugation of internodal segments of etiolated pea seedlings. However, Murphy and O'hEocha[16] found that 'nearly all' the peroxidase activity in the red alga *Cystoclonium purpureum* was in the pellet obtained by centrifuging the homogenate at 100,000 g for 90 min. De Jong,[17] using histochemical methods, concluded that peroxidase is cytoplasmic in juvenile onion root tissue, but associated with the walls of mature cells.

Figure 1 shows the peroxidase isozymes separated by electrophoresis. Cotton peroxidase isozyme No. 5 occurs in old leaves and in the stem. No. 4 occurs in old leaves, stems and boll. Isozyme No. 3 is found in young leaves and old leaves. No. 2 is present in all four tissues examined; while No. 1 is restricted to the boll.

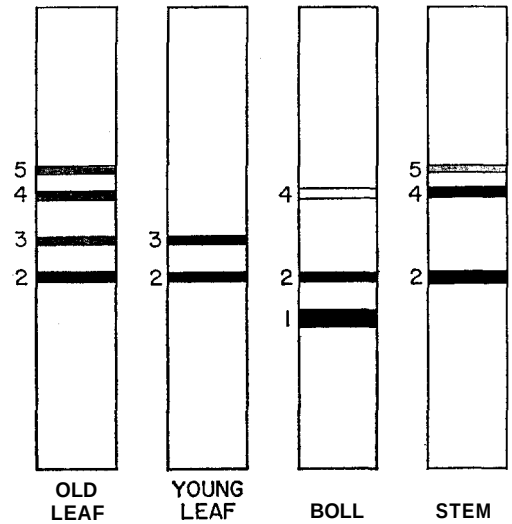

FIG. 1. ACRYLAMIDE GEL DISC ELECTROPHORESIS OF EXTRACTS FROM VARIOUS TISSUES OF THE COTTON PLANT. THE ISOZYMES WERE SEPARATED AND LOCALIZED AS DESCRIBED IN THE TEXT.

There appears to be an increase in peroxidase isozymes with age of the cotton leaf blade. Similar qualitative changes have been reported in corn leaves by Hamill and Brewbaker'[18]

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and in the corn leaf sheath by McCune.[7] However, Alvarez[19] observed no qualitative changes in the peroxidase isozyme pattern during development of the orchid fruit.

There also appears to be a tissue- or organ-specific isozyme pattern in the cotton plant. For example, isozyme No. 3 occurs only in leaves and No. 1 only in the boll. Similarly, Evans[8] observed that the pith, cortex and leaf of the tomato plant each has its own complement of peroxidases, though some were common to all three parts. It has been reported that the barley embryo and the rest of the seed had few peroxidases in common.[9]

It is interesting to note that most plants contain more peroxidase isozymes than the cotton plant.[7-9] In preliminary experiments, using the same techniques for separation, we found thirteen isozymes in the soybean leaf, nine in the radish leaf, and twelve in the radish root. Only five of the radish isozymes were common to both organs examined.

Table 1 indicates that there are significant quantitative differences in the peroxidase activity among the organs of the cotton plant. Figure 1 indicates that different peroxidase isozymes are present in different organs. It is difficult to come to any conclusion concerning the role of peroxidase on the basis of tissue localization alone. However, one might speculate that since isozyme No. 3 occurs only in leaves, it may possibly be associated with a specific function of the leaf. Since No. 2 is present in each of the tissues examined, it may play a more fundamental role in the physiology of the cotton plant than the other peroxidase isozymes. No. 1, found only in the boll, may have a role in reproductive physiology.

EXPERIMENTAL

Plant tissue. Plants of ***Gossypium hirsutum*** L. var. Auburn 57 were field-grown in Shelby County Tennessee, and the mature plants were harvested in early September, 1969. The plant parts used were washed in H_2O. The 'stem' tissue consisted of segments from the middle three internodes of the plant. The entire tap root was used. The 'boll' tissue consisted of the nearly mature green ovary wall, 3-5 cm long. The petiole and the three main veins were removed from each leaf. 'Young leaves' were those obtained from the top three nodes of the plant. 'Old leaves' were the most mature healthy leaves that did not contain noticeable amounts of pigments other than chlorophyll. The various plant tissues were chopped into 0·5–1·0 cm pieces and homogenized in a Waring blender at slow speed for 10 sec, then at max speed for 30 sec with several vol. of cold aq. 1% cholate. The slurry was ground using a Dounce homogenizer prior to assay for total peroxidase activity.

Fractionation of leaf tissue. The mature leaf blade tissue was chopped and homogenized in a Waring blender at max speed for 10 sec with 3-4 vol. of 0·5 M sucrose in 0·02 M glycylglycine, pH 7·5.[13] The crude homogenate was squeezed through 4 layers of cheesecloth. The pH of the filtered homogenate was immediately adjusted to 7.5. Fourparticle fractions and a soluble fraction were isolated by differential centrifugation (Table 2). The homogenate (I) was centrifuged at 180 ***g*** for 20 min. The residue (II) was removed and the supernate was then centrifuged at 6000 ***g*** for 30 min to yield residue III. The supernate from III was centrifuged at 35,000 g for 30 min. After the residue (IV) was obtained the supernate was centrifuged at 100,000 ***g*** for 75 min to give residue V. The five residue fractions were each ground using a Dounce homogenizer in an aqueous mixture containing 1% sodium cholate; the final supernate (VI) was also made 1% with respect to cholate. All manipulations to this point were performed at O-4". The six subcellular fractions were assayed for peroxidase activity and for total protein.[20]

Peroxidase activity. The enzyme was measured by following the oxidation of guaiacol at 470 nm with a recording spectrophotometer using essentially the method of Hosoya and Morrison,[21] except the concentration of H_2O_2 in the reaction mixture was 4·3 mM, the observed optimum concentration for the cotton leaf enzyme. The amount of enzyme which gave an initial reaction rate corresponding to 0·001 optical density units per sec was defined as a milli guaiacol unit (G.U.).

Gel *electrophoresis*. Crude aqueous homogenates prepared from young leaf, old leaf, stem and boll were centrifuged at 35,000 ***g*** for 30 min. Each supernate, which contained over 90% of the peroxidase activity in the whole homogenate, was adjusted to pH 7·5, treated with cholate and centrifuged. The cholate-containing supemate was dialysed overnight against distilled water. An aliquot of the dialysed extract,

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together with brom-phenol blue dye marker in a total volume of 0·1 ml, was placed on a basic acrylamide gel column." One mV per gel column was applied in the cold for 5-10 min, then increased to 2 mV until the dye marker had migrated to the opposite end of the gel. After electrophoresis the peroxidase isozymes were located in the gel using the benzidine stain.[23]

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