Effect of Indoleacetic Acid and Hydroxyproline on Isoenzymes of Peroxidase in Wheat Coleoptiles[1]

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ABSTRACT

Indoleacetic acid at 0.017 millimolar inhibited the formation of three peroxidase isoenzymes in both soluble and wall-bound enzyme fractions of wheat coleoptile (*Triticum vulgare*) tissue. Hydroxyproline at 1 millimolar prevented the indoleacetic acid-induced inhibition. Indoleacetic acid oxidase activity in the soluble fraction was decreased by indoleacetic acid and was restored by hydroxyproline. Most of the indoleacetic acid oxidase activity was located in the electrophoretic zones occupied by two of the peroxidase isoenzymes influenced by indoleacetic acid and hydroxyproline. At least part of the effect of hydroxyproline on auxin-induced elongation of coleoptile tissue may be through control of auxin levels by indoleacetic acid oxidase.

Free hydroxyproline inhibits the elongation of excised coleoptiles, especially the elongation induced by IAA (1, 2). Hydroxyproline has been shown recently to occur in peroxidase of some plant tissues (7, 11). Peroxidase may be involved in coleoptile elongation by its action as an IAA oxidase. These observations have prompted an investigation of the effect of IAA and hydroxyproline on peroxidase activity in wheat coleoptiles.

MATERIALS AND METHODS

All of the experiments utilized coleoptiles of wheat (*Triticum vulgare* L. cultivar Redcoat) grown in the dark in vermiculite for 72 hr. Sections, 9 mm long, were cut 3 mm from the tips, and leaves were removed from the coleoptile cylinders. The sections were incubated in groups of 25 for 20 hr on a shaker at 25 C, in darkness. All solutions contained potassium maleate buffer (pH 4.8, 2.5 mM) and sucrose, 50 mM. IAA, when used, was 0.017 mM and hydroxyproline was 1 mM.

After incubation, the sections were measured and then ground in a mortar with phosphate buffer, pH 7.0, 67 mM. The tissue was extracted with 10 ml of the phosphate buffer for 1 hr in an ice bath. Solid material was separated by centrifugation at 1000*g* for min, and the supernatant was used as the crude, soluble enzyme fraction. This fraction was diluted with 4 volumes of distilled water before peroxidase determinations were made. The tissue was then washed three times with distilled water, which was followed by overnight extraction of

bound peroxidase with 2 ml of 10 mM $Ca(NO_3)_2$ solution (9). The calcium treatment released the bound enzyme which was collected in the supernatant by centrifugation and used for peroxidase determinations without dilution. Peroxidase activity was estimated by measuring the increase of absorbance of a mixture containing 2 ml of 120 mM guaiacol and 5 mM H_2O_2 in 0.02 M phosphate buffer, pH 5.8, to which was added 0.05 ml of enzyme solution.

Peroxidase isoenzymes were separated by starch gel electrophoresis. The procedure given by Ockerse *et al.* (8) was followed, except that the gel was made with 12 g of starch per 100 ml of buffer. Ten milliliters of soluble enzyme solution, extracted as described above from 60 coleoptile sections per treatment, were dialyzed overnight against distilled water. The dialyzed solutions were freeze-dried and then dissolved in 0.6 ml of distilled water. Rectangles of Whatman No. 3MM paper, 4 × 10 mm, were soaked in the solutions and inserted into a 5- × 195- × 195-mm starch plate. The plate was run in a refrigerator for 90 min at 400 v. The surface of the plate was flooded with 120 mM guaiacol and 5 mM H_2O_2 in 0.2 M phosphate buffer, pH 5.8.

Wall-bound enzyme was extracted overnight with 3 ml of $Ca(NO_3)_2$ solution as described above. The extracts were dialyzed as above, freeze-dried, dissolved in 0.1 ml of distilled water, and separated electrophoretically in the same way as the soluble fractions. Soluble and wall-bound peroxidases were extracted from a sample of 60 coleoptile sections just after cutting, to which no treatments were applied. The electrophoresis experiment was repeated three times with similar results.

Samples of the dialyzed soluble and wall-bound fractions prepared for electrophoresis were tested for IAA-oxidase activity. Reaction mixtures contained 0.025 ml of 1 mM 2,4-dichlorophenol, 1 ml of 1 mM IAA in 0.5 mM $MnCl_2$, 3.5 ml of 0.02 M KH_2PO_4, and 0.5 ml of the enzyme solution. One-milliliter samples of the reaction mixtures were withdrawn periodically and were mixed with 2 ml of Salkowski reagent (12). The remaining IAA was estimated colorimetrically at 525 nm.

RESULTS AND DISCUSSION

Hydroxyproline by itself caused nearly a 50% reduction in growth of the coleoptile sections, compared with the control treatment (Table I). IAA-treated sections grew 5.5 mm more than the controls; however, when hydroxyproline was included with IAA, the increase was only 0.7 mm. Thus, as has been found in oat coleoptiles (1), hydroxyproline nearly eliminated auxin-induced growth.

Peroxidase activity was essentially the same in the control and in the hydroxyproline treatments. There was a significant reduction in peroxidase activity of IAA-treated sections from that of the control sections. When hydroxyproline was included

Table I. *Growth and Peroxidase Activity in Wheat Coleoptile Sections Incubated with IAA and Hydroxyproline*

All treatment solutions contained potassium-maleate buffer, buffer, pH 4.8, 2.5 mM, and sucrose, 50 mM. Concentration of IAA was 0.017 mM; hydroxyproline, 1 mM. Samples of 25 coleoptile sections were incubated for 20 hr at 25 C in 3 ml of solution in darkness. Peroxidase reaction mixture contained 2 ml of 120 mM guaiacol and 5 mM H_2O_2, to which was added 0.05 ml of enzyme solution. Standard errors follow treatment means. Peroxidase extracted from the 9-mm-long sections before incubation in treatment solutions gave ΔA min·25 sections = 160 and 2 for soluble and wall-bound solutions, respectively.

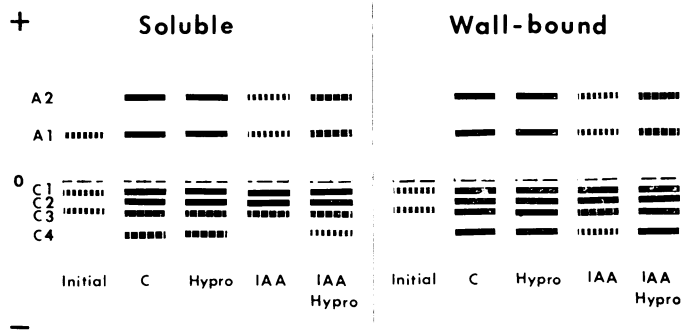| Treatment	Average Increase in Length of Coleoptile Sections	Peroxidase Activity	
		Soluble	Wall-bound
	mm	$\Delta A/min$·25 coleoptile sections	
Control	7.5 ± 0.8	333 ± 60	9.6 ± 2.0
Hydroxyproline	4.2 ± 0.4	333 ± 50	10.1 ± 1.6
IAA	13.0 ± 0.9	200 ± 13	6.0 ± 0.8
IAA + hydroxyproline	8.2 ± 0.6	295 ± 22	10.9 ± 1.4

FIG. 1. Diagram of peroxidase isoenzymes from soluble and wall-bound enzyme fractions separated by starch gel electrophoresis. Initial samples were made from sections freshly cut from 3-day-old coleoptiles. Other samples were from sections incubated for 20 hr in the following media: Control (C): potassium maleate buffer, pH 4.8, 0.05 M sucrose; Hypro: C + 1 mM hydroxyproline; IAA: C + 0.017 mM IAA; IAA-hypro: C + 0.017 mM IAA + 1 mM hydroxyproline. Shading of the bands represents intensity of reaction with guaiacol and H_2O_2.

with IAA, however, the peroxidase activity was restored to near the control level. The decrease in activity caused by IAA was greater in the wall-bound fraction than in the soluble fraction.

Electrophoresis showed that the soluble enzyme fraction of the control treatment (incubated without IAA or hydroxyproline) contained two isoenzymes which moved toward the anode and four which moved toward the cathode. These isoenzymes were designated A1, A2, C1, C2, C3, and C4 (Fig. 1). All six isoenzymes were present in the sample incubated in hydroxyproline without IAA. In the sample incubated in IAA alone, isoenzymes A1 and A2 were greatly diminished, and C4 was absent. All of these were evident in the IAA-hydroxyproline treatment. Isoenzymes A2, C2, and C4 were absent in the initial sample which was extracted before treatments were applied. These three isoenzymes thus increase during the 20-hr incubation period so long as IAA is not included in the medium. The formation of A1, A2, and C4 was inhibited by IAA during incubation. If hydroxyproline is included with IAA, inhibition of these isoenzymes is partially overcome, but their

concentrations are still lower than those formed in the absence of IAA. Repression of peroxidase isoenzymes by IAA has been reported previously (4, 8). It is not possible from the data of this study to decide whether the IAA effect on peroxidase is repression or inhibition. There is no doubt that the activity of certain isoenzyme bands on guaiacol is reduced by IAA; however, this could be the result of less enzyme or of partial inhibition of those enzymes.

The six isoenzymes of the soluble fraction were also present in the wall-bound fraction; however, the distribution of their activities was different from those in the soluble fraction (Fig. 1). A1 was absent from the initial sample, but C1 and C3 were present, as in the soluble fraction. A1, A2, and C4 were repressed (or diminished) by IAA alone, but hydroxyproline included with IAA caused a partial reversal of the repression, as in the soluble fraction. The major differences between the soluble and wall-bound isoenzymes were in C4. C4 in the wall-bound fraction was much more apparent than in the soluble fraction. The repressing effect of IAA and the reversal of IAA-induced repression by hydroxyproline were similar to the effects in the soluble fraction.

Neither IAA nor hydroxyproline appear to have much effect on isoenzymes C1, C2, and C3 in either fraction.

The activity of IAA oxidase paralleled peroxidase activity in the crude soluble enzymes. The rate of auxin destruction was 38 μg/25 coleoptile segments per min in control, hydroxyline, and hydroxyproline-IAA treatments. In the IAA treatment, the rate of auxin destruction was 31 μg/min. The freshly cut untreated sections had an activity of 11 μg of auxin destroyed per 25 sections per min. No IAA oxidase activity was found in the wall-bound fraction.

Electrophoretic separations had shown that certain peroxidase isoenzymes were sensitive to IAA and hydroxyproline and others were not. A determination was then made to see whether IAA oxidase activity was associated with the sensitive peroxidase isoenzymes.

Soluble enzyme solutions were prepared as in the Materials and Methods section from two groups of 100 coleoptiles each. One group had been incubated with IAA alone, and the other with both IAA and hydroxyproline. The solutions were dialyzed, then freeze-dried. Resulting powders were mixed with

Table II. *IAA Oxidase Activity Associated with Peroxidase Isoenzymes*

Electrophoretic separations were made of soluble enzyme from 100 coleoptile sections incubated in (1) 0.017 mM IAA and (2) 0.017 mM IAA and 1 mM hydroxyproline under conditions same as for Table I. Peroxidase isoenzymes were located with paper strips saturated with guaiacol-H_2O_2. IAA oxidase was measured in solutions centrifuged from starch gel zones. Reaction mixtures contained 0.025 ml of 1 mM 2,4-dichlorophenol, 1 ml of 1 mM IAA in 0.5 mM $MnCl_2$, 3.0 ml of 0.02 M KH_2PO_4, and 1 ml of enzyme solution from starch gel. IAA destruction was estimated by reacting 1 ml of mixture with 2 ml of Salkowski reagent and comparison of absorbance at 525 nm with standard curve. All values are based on 100 coleoptile sections.

Peroxidase Isoenzyme	(1) IAA	(2) IAA and Hydroxyproline
	μg IAA destroyed/hr	
A1	38	84
A2	5	47
C1	16	36
C2	3	4
C3	3	0
C4	0	7

10 mg of dry starch, and enough water was added to make a slurry. Each slurry was pipetted into a 1-mm-wide slit, 160 mm long, in a starch plate. Electrophoresis was run under conditions described earlier. After the run, paper strips soaked in the guaiacol-H_2O_2 mixture were applied perpendicularly to both ends of the slit, thereby covering 25 mm of the slit at the ends. This procedure caused the formation of visible isoenzyme bands as markers. The unreacted center part of the gel was divided into sections corresponding to the six visible bands. The sections were removed from the plate, were frozen and thawed, and then were centrifuged in fritted glass extraction thimbles inserted into centrifuge tubes in order to obtain solutions. One-milliliter samples of the solutions were tested for IAA oxidase activity as described previously.

IAA oxidase activity in the starch sections was weak, possibly because of partial inactivation by freeze-drying (10). However, after 6 hr of reaction, differences among the starch gel zones were apparent (Table II). The greatest activity was found in band A1 in both samples. The IAA-hydroxyproline treatment yielded twice the activity in A1 as the IAA treatment. The greatest difference between treatments was observed in the A2 band, in which there was a near 10-fold increase when hydroxyproline was included. Band C1 was similar in activity to A1. Much of the IAA oxidase activity was thus associated with the two anodic peroxidase bands, both of which were greatly influenced by IAA and hydroxyproline treatments.

There is no proof that the peroxidase isoenzymes, as detected by guaiacol, are identical with IAA oxidase isoenzymes. Sequeira and Mineo (10) have given evidence that IAA oxidase can occur as a discrete form with no peroxidase activity. However, Galston et al. (4) consider that peroxidase and IAA oxidase activity occur in the same enzyme.

It has been shown that IAA caused a decrease in IAA oxidase activity, and that hydroxyproline prevented the decrease to a great extent. Further, much of the IAA oxidase activity was associated, in electrophoretic mobility, with the two isoenzymes most affected by IAA and hydroxyproline. This relationship suggests that the effect of hydroxyproline on auxin-induced elongation is at least partially through control of auxin levels by IAA oxidase. Although hydroxyproline inhibited significantly the endogenous growth of decapitated wheat coleoptile sections, it did not cause an increase in peroxidase or IAA oxidase. It thus appears that if hydroxyproline inhibits growth

through IAA oxidase, only the IAA-induced growth is so affected. Inhibition of endogenous growth must be explained in some other way.

The manner in which hydroxyproline prevents the IAA-induced decrease in peroxidase and IAA oxidase activity is unknown. Hydroxyproline apparently is a constituent of peroxidase (7, 11), but it is not known whether it is necessary for activity of the enzyme. Hydroxyproline is incorporated directly into protein when it is present at growth-inhibiting levels (3, 5), and some may be incorporated into peroxidase. It has been suggested that hydroxyproline may render certain cell wall proteins nonfunctional by replacing proline, thereby inhibiting growth (3, 6). Although this may be the case in structural, hydroxyproline-rich wall proteins, it does not seem to apply to soluble or wall-bound peroxidase. In these enzymes, hydroxyproline either has no effect or it prevents the repressive effect of IAA.

LITERATURE CITED

1. CLELAND, R. 1967. Inhibition of cell elongation in *Avena* coleoptile by hydroxyproline. Plant Physiol. 42: 271–274.
2. CLELAND, R. AND A. M. KARLSNES. 1967. A possible role of hydroxyproline-containing proteins in the cessation of cell elongation. Plant Physiol. 42: 669–671.
3. CLELAND, R. AND A. C. OLSON. 1967. Metabolism of free hydroxyproline in *Avena* coleoptiles. Biochemistry 6: 32–36.
4. GALSTON, A. W., S. LAVEE, AND B. Z. SEIGEL. 1968. The induction and repression of peroxidase isoenzymes by 3-indoleacetic acid. *In:* F. Wightman and G. Setterfield, eds., Biochemistry and Physiology of Plant Growth Substances. Runge Press, Ottawa, p. 455–472.
5. HOLLEMAN, J. 1967. Direct incorporation of hydroxyproline into protein of sycamore cells incubated at growth inhibitory levels of hydroxyproline. Proc. Nat. Acad. Sci. U.S.A. 57: 50–54.
6. LAMPORT, D. T. A. 1970. Cell wall metabolism. Annu. Rev. Plant Physiol. 21: 235–270.
7. LIU, E. H. AND D. T. A. LAMPORT. 1968. An hydroxyproline-O-glycosidic linkage in an isolated horseradish peroxidase isozyme. Plant Physiol. 43: S-16.
8. OCKERSE, R., B. Z. SIEGEL, AND A. W. GALSTON. 1966. Hormone-induced repression of a peroxidase isozyme in plant tissue. Science 151: 452–453.
9. PARISH, R. W. AND F. L. MILLER. 1969. The uptake and effects of calcium and phosphate on maturity, lignification, and peroxidase activity of wheat internodes. Aust. J. Biol. Sci. 22: 77–85.
10. SEQUEIRA, L. AND L. MINEO. 1966. Partial purification and kinetics of indoleacetic acid oxidase from tobacco roots. Plant Physiol. 41: 1200–1208.
11. SHANNON, L. M., E. KAY, AND J. Y. LEW. 1966. Peroxidase isozymes from horseradish roots. J. Biol. Chem. 241: 2166–2172.
12. TANG, Y. W. AND J. BONNER. 1947. The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. Arch. Biochem. 13: 11–25.