

Ethylene-induced Phenylalanine Ammonia-Lyase Activity in Carrot Roots^{1, 2}

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ABSTRACT

Ethylene enhanced the activity of phenylalanine ammonia-lyase in carrot (*Daucus carota* L., var. "Nauty") root tissue. Slight increase in enzyme activity was exhibited by root discs incubated in ethylene-free air. It was probably due to the ethylene formed within the sliced tissue. Addition of ethylene to the air stream increased phenylalanine ammonia-lyase activity and the total protein content of the discs until maximum activity was reached after 36 to 48 hours of incubation. The continuous presence of ethylene was required to maintain high level of activity. Ethylene, at a concentration of 10 microliter per liter induced higher activity than at lower or higher concentrations. CO₂ partially inhibited the ethylene-induced activity. Cycloheximide or actinomycin D effectively inhibited the ethylene-induced activity in discs that had not previously been exposed to ethylene. The results appear to support the hypothesis that the mode of action of ethylene may involve both *de novo* synthesis of the enzyme protein and protection or regulation of activity of the induced enzyme.

MATERIALS AND METHODS

Fresh carrot (*Daucus carota* L., var. "Nauty") roots of uniform size were washed in detergent and water and surface sterilized with 70% ethanol. Uniform discs (1 cm thick and 1.5 cm in diameter) were cut from the roots and placed on moist filter paper in open Petri plates. About 50 discs were incubated at 20 C in the dark, inside 20-liter glass jars through which air, ethylene-air, and other gaseous mixtures saturated with water were continuously passed at a rate of 100 ml/min.

For nucleic acid and protein synthesis inhibitor studies, discs were dipped for 1 min into the inhibitor solution, then placed on filter paper in Petri plates which contained 2 ml of the inhibitor solution.

Acetone powders were prepared from the root discs by cutting the tissue into small pieces and placing 50-g tissue in 500 ml of acetone previously chilled to -18 C. The acetone was changed twice within a period of 1 hr. The tissue was then homogenized for 3 min, filtered through a Whatman No. 1 filter paper in a Buchner funnel, and rinsed with two 500-ml portions of chilled acetone. Mats were dried for 1 hr at room temperature, then kept overnight under vacuum, and stored at -18 C.

PAL activity in acetone powders was assayed by the method of Rahe *et al.* (14) with slight modifications. PAL was extracted by suspending 0.5 g of powder in 10 ml of cold 0.1 M borate buffer, pH 8.8. The suspension was kept in an ice bath for 1 hr with occasional stirring and centrifuged at 40,000g for 20 min at 1 C. The supernatant served as the enzyme preparation. For the assay, 1.5 ml of the supernatant was added to a test tube containing 1.0 ml of 0.05 M L-phenylalanine and 2.5 ml of 0.1 borate buffer, pH 8.8. The assay mixture was incubated in a water bath at 40 C for 1 hr, the reaction was stopped by the addition of 0.1 ml of 5 N HCl, and the acidified mixture was extracted with 7 ml of ether. Two and one-half ml of the ether phase were evaporated to dryness, and the residue was dissolved in 2.5 ml of 0.05 N NaOH. The concentration of cinnamic acid was determined by absorbance at 269 nm, with 0.05 N NaOH as reference. Two controls were run in each assay; a no-substrate assay in which 1.0 ml of deionized water was substituted in the reaction mixture for the L-phenylalanine solution and a zero time stop reaction assay mixture acidified at zero time of incubation. Both controls were assayed as described above. Values obtained from the zero time incubation were applied in the determination of PAL activity. Linearity of the rate of cinnamic acid production during the 1-hr incubation period was verified by acidifying assay mixtures at various times during incubation. A unit of activity was defined as that amount of enzyme which catalyzes the production of 1 μ mole cinnamic acid/hr. Specific enzyme activity was expressed as units/mg protein. Soluble protein content was de-

Considerable effort has been devoted in recent years toward a better understanding of the physiological effects of ethylene in plants (13). One approach aimed at elucidating mechanisms whereby ethylene exerts its effects on plant tissues has been to investigate its effect on RNA and protein synthesis (1), and on the level and activity of various enzymes (5, 7, 9, 10, 17, 19). In these studies evidence was presented for the induction by ethylene of *de novo* synthesis of enzymes and increased enzymatic activity.

L-Phenylalanine ammonia-lyase (EC 4.3.1.5.) may play an important regulatory role (12) by its effect on the rate of the initial reaction in the pathway from phenylalanine to a wide variety of plant phenols. The activity of this enzyme in plants is affected by various treatments and environmental conditions, including ethylene treatment (4, 8, 10, 15, 16).

This paper presents data on the induction by ethylene of PAL³ activity in carrot root tissue. Some aspects of the kinetics of this induction are also discussed.

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³ Abbreviation: PAL: phenylalanine ammonia-lyase.

terminated by the biuret method (6). All experiments were replicated at least twice.

RESULTS

As illustrated by a typical experiment (Fig. 1), PAL activity in carrot root discs was increased by ethylene treatment. In freshly harvested roots, activity was always low and ranged from 0 to 4 units of enzyme/mg protein. Discs incubated in air showed a slight increase in activity during the first 24 hr of incubation. Incubation in an air stream, containing ethylene at various concentrations, showed maximum PAL activity after 36 to 48 hr. Longer incubation periods resulted in lower ac-

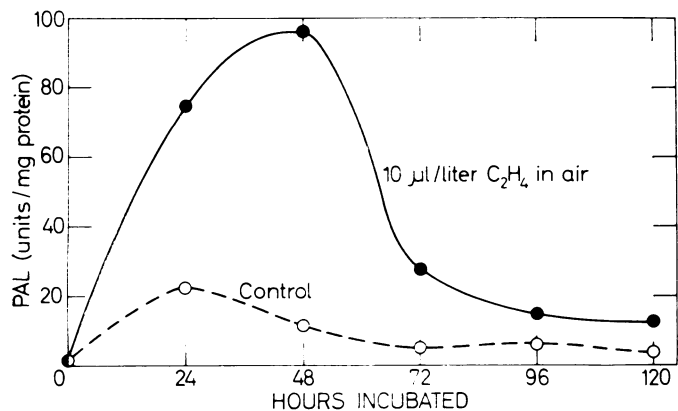


FIG. 1. Effect of ethylene on the induction of PAL activity in carrot root discs. About 50 uniform discs (1 cm thick and 1.5 cm in diameter) were incubated for various periods of time at 20 C in the dark inside 20-liter glass jars through which air (control) or 10 µl/liter C₂H₄ in air was continuously passed at a rate of 100 ml/min.

Table I. Protein Content of Carrot Root Discs Incubated in an Air Stream Containing Ethylene

Incubation Period	Air Control	Treated with 10 µl Ethylene/liter of air
<i>hr</i>	<i>mg ml of enzyme preparation</i>	
0	18.6	18.6
24	21.3	21.9
48	20.6	28.7
72	23.0	30.3
96	22.0	31.2

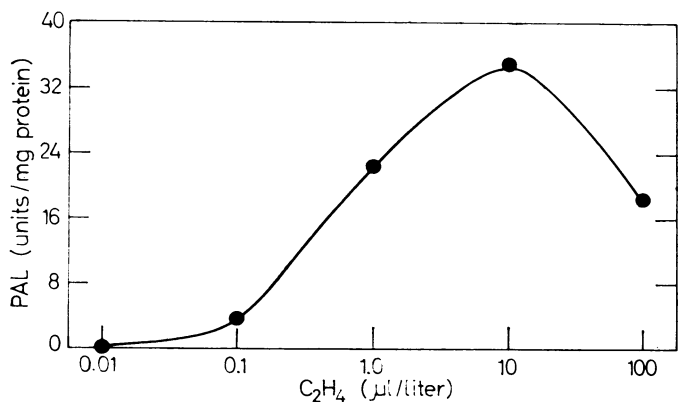


FIG. 2. Effect of ethylene concentrations on induction of PAL activity in carrot root discs. Discs were incubated for 48 hr at various concentrations of ethylene. Other experimental conditions as in Figure 1.

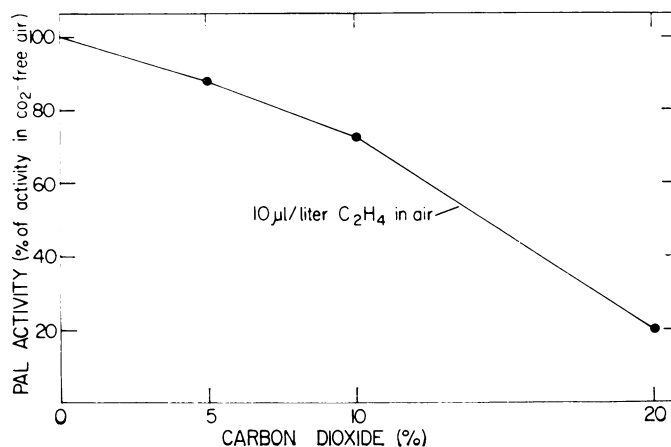


FIG. 3. Effect of CO₂ concentrations on ethylene-induced PAL activity in carrot root discs. Discs were incubated for 48 hr at 5, 10, and 20% CO₂ concentrations. Other experimental conditions as in Figure 1. Activity at 100% of control was 38 units of enzyme/mg of protein.

tivity. Ethylene treatment also increased the total protein content (Table I) of carrot root discs. Therefore, by expressing PAL activity on the basis of protein content, the difference between discs incubated in ethylene and those incubated in air is reduced, particularly at long incubation periods.

The effect of ethylene concentrations on the induction of PAL activity is shown in Figure 2. Maximum induction was obtained at 10 µl/l ethylene. Incubation at 1000 and 10,000 µl/l ethylene resulted in activities lower than 10 units of enzyme/mg of protein.

The continuous presence of ethylene was required to maintain high PAL activity in the discs; when ethylene was withdrawn from the air stream at any time during the first 48 hr of incubation, PAL activity decreased within less than 24 hr to a level close to that of the control. When ethylene was reintroduced, activity increased.

Figure 3 shows the effect of increasing CO₂ concentrations on ethylene-induced PAL activity in carrot root discs. At 20% CO₂, PAL activity in the discs was markedly inhibited. Upon addition of 100 µl/l ethylene to the air stream which contained 20% CO₂, the inhibitory effect was partly relieved; PAL activity increasing from 20% (Fig. 3) to 45% of air control.

The effect of protein and nucleic acid inhibitors on ethylene-induced PAL activity is shown in Figure 4. Dipping carrot root discs for 1 min in a 15 µg/ml solution of cycloheximide at zero time of incubation, inhibited by more than 90% the induction of PAL activity by ethylene. Application of the same concentration of cycloheximide after 24 hr of incubation failed to inhibit PAL activity to the same extent, and application after 48 hr of incubation had no inhibitory effect at all (treatment A, Fig. 4). Inhibition by more than 65% was also noted when discs incubated for 48 hr in air were treated with the inhibitor solution and then incubated for an additional 24 hr period in ethylene (treatment B, Fig. 4). However, inhibition was less effective (only 30%) in discs incubated for 24 hr in ethylene, followed by a 24-hr incubation period in air, and then treated with the inhibitor solution and retransferred to ethylene (treatment C, Fig. 4).

Similar results on the inhibition of ethylene-induced PAL activity in carrot roots were also obtained when discs were dipped in a 50 µg/ml solution of actinomycin D.

DISCUSSION

The data presented here on the induction of PAL activity by ethylene in carrot root discs are similar to those reported for

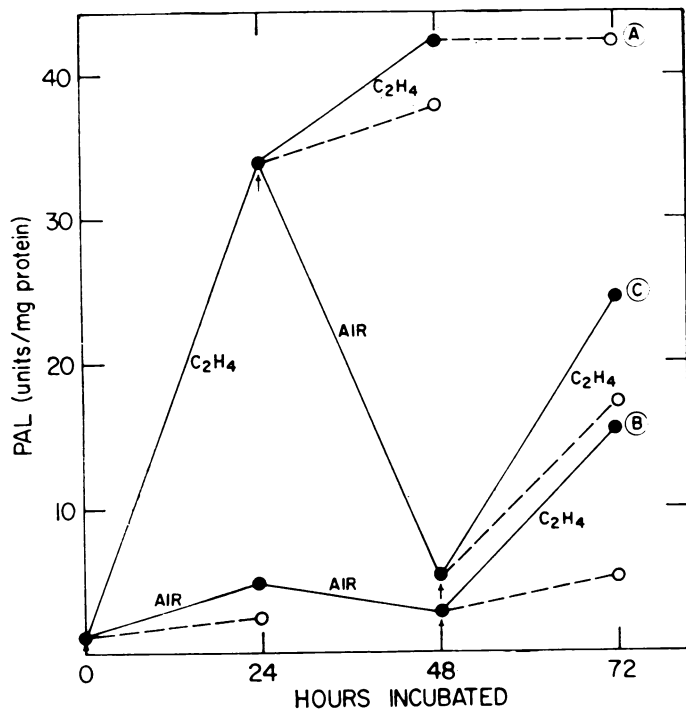


FIG. 4. Effect of addition and withdrawal of ethylene and of applications of cycloheximide on PAL activity in carrot root discs. At 0, 24 and 48 hr of incubation (as indicated by arrows), discs were dipped for 1 min in a cycloheximide solution ($15 \mu\text{g}/\text{ml}$) and then replaced in the incubation jar. \circ : PAL activity 24 hr after cycloheximide treatment. Other experimental conditions as in Figure 1.

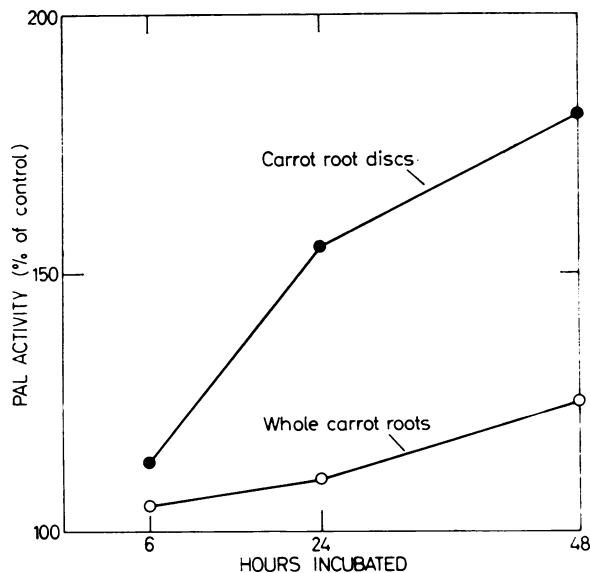


FIG. 5. Ethylene induction of PAL activity in carrot root discs and in whole carrot roots. Carrot discs and whole carrots were incubated inside the same jars. Other experimental conditions as in Figure 1.

grapefruit (16), for pea seedlings (8), and for swede and parsnip root tissues (15). They do, however, differ in one important aspect from recently reported findings (4), showing that ethylene induced PAL activity in excised gherkin hypocotyl tissue but inhibited PAL in intact hypocotyls. As in intact grapefruit (16) and intact swede roots (15), ethylene induced

PAL activity in intact carrot roots as it did in discs but at a slower rate (Fig. 5). In carrot root tissue, PAL activity is induced mostly in the newly formed phloem cells. This region comprised a larger percentage of the sampled tissue in discs than in intact roots. Moreover, the slicing of the roots into discs also caused a slight induction of PAL activity (Fig. 1, control). Therefore, the discs would be expected to exhibit higher PAL activity than intact roots.

The slight increase in PAL activity and in protein content noticed in carrot root discs incubated in air (Fig. 1, Table I), may have been induced by the ethylene produced by the discs as the result of wounding. During the first 24 hr of incubation, carrot root discs produced ethylene at a rate of 0.04 to $0.1 \mu\text{l}/\text{kg}\cdot\text{hr}$, whereas at longer incubation periods the rate of ethylene production decreased to $0.04 \mu\text{l}/\text{kg}\cdot\text{hr}$ (2). Since these concentrations of ethylene induced low activity in carrot root discs (Fig. 2), it is possible that the slight induction of PAL activity and the increase in protein content in the discs incubated in air may be due to the higher rate of ethylene production during the first 24 hr of incubation, resulting from the slicing of the tissue.

The data presented in Figure 4 suggest that induction of PAL activity by ethylene may be the result of *de novo* synthesis of the enzyme protein in tissue which initially exhibited a low rate of enzyme activity or none at all. Figure 4 also shows, however, that the continuous presence of ethylene was required for maintaining a high level of activity. Also the effectiveness of the protein and nucleic acid inhibitors was reduced in tissues that either exhibited (treatment A) or had exhibited (treatment C) high PAL activity possibly demonstrating its requirement at the phase of enzyme induction. These results may support the hypothesis (2, 13, 18) that the presence of ethylene is not only required for inducing synthesis of the enzyme protein at which phase the inhibitor effectively reduced PAL activity, but also for maintaining continuous high activity of the induced enzyme. At the latter instance, the enzyme protein may have already been synthesized, and therefore effectiveness of the inhibitor was reduced. Nevertheless, presence of ethylene was still required. Ethylene may combine with the induced enzyme or protect it from inactivation, or both. No evidence was obtained of any direct and immediate effect of ethylene on PAL activity in enzyme preparations from discs incubated for various periods in ethylene, air, or intermittently in both media.

The decrease of PAL activity after 48 hr in the continued presence of ethylene, as noted in carrot root discs (Fig. 1) and in other plant tissues (8), may be attributed either to the cell regulatory mechanism, which inactivates PAL (3, 20) or to feedback inhibition (11). It has been assumed (8, 20) that there exist both a PAL-synthesizing and a PAL-inactivating system, and that in the course of prolonged incubation, PAL activity declines, as the inactivating system becomes active and finally dominant. While this assumption may aid in the understanding of changes in PAL activity in carrot root discs, in this tissue the rate of decrease in PAL activity at longer than 48 hr of incubation was not affected by the presence of ethylene during the period of declining activity.

The present experiments confirm and extend the knowledge on induction of PAL activity by ethylene. While no clear indication of the way in which ethylene causes this induction is rendered, some evidence is presented to suggest that, in addition to inducing synthesis of the enzyme protein, ethylene may also regulate the activity of the induced enzyme, as indicated by the time-course inhibition studies.

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