Induction of Phenylalanine Ammonia-lyase in Strawberry Leaf Disks

ACTION SPECTRA AND EFFECTS OF WOUNDING, SUCROSE, AND LIGHT

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ABSTRACT

The increase in phenylalanine ammonia-lyase (PAL) activity in strawberry (Fragaria vesca var. WSU-1232) leaf disks required wounding, sucrose, and light and was cycloheximidesensitive. In injured leaves and in leaf disks, the highest PAL activity was detected nearest the wounded tissues. Without wounding, no increase in activity was observed when leaves were cultured in sucrose and light.

The optimal concentration of sucrose for enzyme activity increase ranged from 0.15 M to 0.4 M. At the suboptimal sucrose concentration, the level of PAL activity was dependent upon the concentration of sucrose. A low but constant level of activity was detected in leaf disks maintained in 0.15 M sucrose and in darkness. Light accelerated the rate of PAL increase but did not change the total level of enzyme activity which was determined by the sucrose concentration.

Enzyme activity disappeared rapidly when leaf disks cultured in sucrose and light were transferred to darkness or to water in light. Unlike in Xanthium leaf disks, cycloheximide could not completely inhibit the decay of enzyme activity, suggesting that an inactivating system was synthesized during the induction period, and the activity of the inactivating system increased as the induction period lengthened.

The effect of light on accumulation of PAL activity appeared to be linked to photosynthesis. In the presence of 25 μM 3-(3,4-dichlorphenyl)-1,1-dimethylurea, the effect of light on enzyme increase was completely nullified. Addition of 25 μM 3-(3,4-dichlorphenyl)-1,1-dimethylurea to culture medium caused rapid decay of PAL activity from leaf disks which had been previously cultured in sucrose and light. The relation between effect of light and photosynthesis was further demonstrated by the action spectrum. Leaf disks incubated in sucrose and light of different wavelengths exhibited maximum accumulation of PAL activity at two wavelengths (475 nm and 625 nm). Action spectrum for protection against PAL decay exhibited a plateau at 475 to 525 nm and a peak at 625 nm. Action spectra for accumulation and protection against inactivation of PAL activity, therefore, appeared to be very similar to the action spectrum of photosynthesis.

L-Phenylalanine ammonia-lyase (EC 4.3.1.5) is the first and limiting enzyme in the pathway of phenolic biosynthesis (7, 18). It catalyzes the deamination of L-phenylalanine to form L-cinnamic acid and ammonia. Cinnamic acid is a precursor for many plant secondary constituents. PAL was first discovered by Koukol and Conn (13) and has since been found in a wide variety of plants (4). This enzyme has been of interest not only for its role in plant phenolic metabolism, but because its activity fluctuates significantly in plant tissues in response to a variety of physical and chemical stimuli (4, 20). PAL has become a model for studies of the mechanism of enzyme turnover.

The present report demonstrates that wounding and incubating strawberry leaf disks in sucrose and light significantly increased the PAL activity. The results show that the total level of PAL activity is dependent on the sucrose concentration of the culture medium and that light accelerated the rate of PAL increase. The stimulatory effect of light appeared to be linked to photosynthesis.

MATERIALS AND METHODS

Chemicals and Source of Plant Materials. Reagent grade chemicals or those of the highest grade available were obtained from commercial sources. Mercaptoethanol, cycloheximide, and L-phenylalanine were purchased from Sigma Chemical Co. (St. Louis, Mo.), and DCMU was purchased from Aldrich Chemical Co. (Milwaukee, Wisc.). Strawberry (Fragaria vesca var. WSU-1232) plants were grown in the greenhouse with supplemental fluorescent lights. A light period of 16 hr was maintained.

Experimental Conditions. Except where indicated, experiments were carried out with leaf disks 1.3 cm in diameter which were cut from the lamina of the youngest fully expanded leaves. Injury was inflicted upon the disks by pressing

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2 Milton Zucker died in July, 1973. This paper is his final contribution to science. Dr. Zucker was a good and dedicated scientist. His friendship and his guidance will be long cherished.

3 Abbreviation: PAL: phenylalanine ammonia-lyase.
them against a plastic shading plate (Technygraph Co., Techny, Ill.). Leaf disks from a number of leaves were pooled in each experiment. Randomly chosen sets of 15 disks were washed in 0.1 M sucrose and were cultured on filter paper floating on 15 ml of 0.1 M sucrose in disposable Petri dishes. The sucrose solution was changed every 24 hr until the end of the experiment. Cultures were maintained at 23 to 25 C under 600 ft-c of light in a growth chamber (National Appliance Co., Portland, Ore.). Light intensity was varied by placing the dishes at different distances from the light source. Dishes were wrapped in aluminum foil for culture in darkness. After culturing, the disks were used either immediately for PAL assay or frozen in liquid nitrogen and stored at -20 C for later use.

For the studies on wounding effects, a leaf disk 2.2 cm i.d. was cut and cultured in sucrose and light for 60 hr. PAL was assayed in relation to the distance away from the edge (wound). In other experiments, a wound 1 cm i.d. was inflicted on a detached leaf by pressing it against a plastic shading plate. After culturing in sucrose and light for 60 hr, PAL was assayed in relation to the distance away from the wound. In the third experiment, a doughnut-shaped wound 2.4 cm o.d. and 1.8 cm i.d., was made on a detached leaf. The leaf was incubated in sucrose and light for 60 hr; PAL was assayed in relation to the distance away from the wound and toward the center of it.

The action spectra for induction and inactivation of PAL were determined with a large spectrograph capable of producing a 16-ft long continuous spectrum. Details of the structure and operation of the spectrograph were described by Balegh and Biddulph (2). Leaf disks used in determining the induction spectrum were cut and injured as described and incubated in 0.1 M sucrose under the light source with wavelength ranging from 350 to 740 nm. After 33 hr of incubation, PAL activity was assayed. For the determination of the inactivation spectrum, leaf disks were preincubated in 0.1 M sucrose under white light for 34 hr to induce PAL accumulation and were then transferred to the spectrograph station where the disks were irradiated at different wavelengths of light for another 24 hr. PAL was assayed and activity compared to the sample which had been cultured in darkness after the preincubation period.

Enzyme Extraction and Assay. PAL was extracted from washed samples of three leaf disks by homogenizing them in a mortar and pestle with 5 ml of cold 25 mM borate-HCl buffer, pH 8.8, containing 0.5 mM β-mercaptoethanol. The homogenate was centrifuged at 10,000g for 15 min. The clear yellow-green supernatant obtained was used for PAL assay.

The enzyme was assayed by a spectrophotometric method as described previously (19). The reaction mixture contained 100 μmoles of borate at pH 8.8, 15 μmoles of L-phenylalanine, 1.5 μmoles of mercaptoethanol, and 0.1 to 0.2 ml of enzyme extract in a final volume of 3 ml. The reaction mixture was preincubated at 30 C for 15 min, and the rate of enzymatic reaction was measured at 30 C in a Gilford recording spectrophotometer by following the linear increase in absorbance at 290 nm over a period of 30 to 45 min.

One unit of activity is defined as that amount of enzyme catalyzing the formation of 1 μmole of L-cinnamic acid from L-phenylalanine in 1 min at 30 C. Most data are reported as milliunits (mU) of enzyme per leaf disk.

RESULTS

Effect of Wounding on Induction of PAL. Wounded strawberry leaves cultured in light and sucrose exhibited a striking increase in PAL activity (Fig. 1). Injury appears to be a necessary requirement for the induction of PAL, since leaves cultured in light and sucrose do not accumulate PAL without wounding. Figure 1 shows that PAL activity is higher near the area of injury than it is in the area farther away from it. Wounded detached leaves produce significant PAL activity; however, no PAL activity was detected in tissue 1 cm away from the wound. When a larger doughnut-shaped wound was made, PAL activity was detected at 0.8 cm away from the wound but no PAL activity was observed at 2 cm away. When PAL activity was assayed toward the center of a leaf disk cultured in light and sucrose, the activity decreased in the tissue away from the injured edge. However, at the center, PAL activity showed an increase (Fig. 1).

Effect of Sucrose Concentration on Accumulation of PAL Activity. Induction of PAL activity in strawberry leaf disks required relatively high levels of sucrose. In other experiments, not shown here, the optimal concentration of sucrose for induction was found to be between 0.15 M to 0.4 M. Figure 2 shows that the level of enzyme activity was a function of the sucrose concentration. At 0.15 M sucrose, PAL activity showed a rapid increase and then a rapid decline after 48 hr of incuba-

![Fig. 1. Effect of wounding on the accumulation of PAL activity. A disk shaped wound was inflicted on a detached strawberry leaf (○) and after incubation in light and sucrose, PAL activity was assayed in the tissue away from the wounded area. A large leaf disk was cultured and PAL activity was assayed in the tissue toward the center of the disk (▲). The distance from the edge of a disk toward its center is designated with a minus sign. A doughnut-shaped wound, 2.4 cm o.d. and 1.8 cm i.d., was inflicted on a detached strawberry leaf (□), and following incubation, PAL activity was assayed away from the outer rim (distance designated with plus sign) and toward the center of the inner rim (distance designated with minus sign). No PAL activity was detected in detached leaf maintained in light and sucrose (shaded area). The value for PAL activity reported here is an average value of duplicate samples. All experiments were repeated twice and similar results were obtained.](https://www.plantphysiol.org/doi/10.1104/pp.54.3.660)
tion. At lower sucrose concentration, PAL activity reached a lower peak, but it should be noted that the time needed to reach the maximum peak of PAL activity at all levels of sucrose was similar (48–52 hr). Without sucrose, no PAL activity was induced (Fig. 2). The increase in PAL activity in the presence of sucrose at any concentration was inhibited by addition of cycloheximide (20 µg/ml). Thus, the increase in activity may result from de novo PAL synthesis.

**Effect of Light Intensity on Accumulation of PAL Activity.**

A significant increase in PAL activity occurred in strawberry leaf disks when maintained in light as compared to those maintained in darkness. However, the levels of PAL activity were proportional to sucrose concentration and not to light intensity. Figure 3 shows that at a constant sucrose concentration, the maximum level of PAL activity achieved was the same whether the leaf disks were exposed to light intensity of 600 ft-c or 100 ft-c. The time courses of PAL synthesis at these two light intensities were different. Leaf disks maintained at 100 ft-c of light at two levels of sucrose reached the maximum PAL activity about 24 hr later than those leaf disks maintained at 600 ft-c (Fig. 3). Although levels of PAL activity are independent of intensity of light, to reach the level of enzyme activity at a given sucrose concentration, light is required. Leaf disks incubated in 0.15 M sucrose in darkness showed low PAL activity after 97 hr of incubation (Fig. 3). There was no indication to suggest that the PAL activity would increase any further even after 120 hr of incubation. Figure 3 shows again that without sucrose no PAL activity was detected in leaf disks which were irradiated at light intensity of 600 ft-c.

![Fig. 2. Time course of PAL activity accumulation at different concentrations of sucrose. Strawberry leaf disks were maintained in light (600 ft-c) and at different levels of sucrose: (1) 0.15 M; (2) 0.03 M; (3) 0.005 M; and (5) no sucrose. PAL activity was assayed at various time intervals as indicated. The value for each point is an average of triplicate samples.](image1)

![Fig. 3. Time course of PAL activity accumulation at different light intensities and at different sucrose concentrations. Strawberry leaf disks were maintained at various light intensities and sucrose concentrations: (1) 600 ft-c and 0.15 M; (2) 100 ft-c and 0.15 M; (3) 600 ft-c and 0.015 M; (4) 100 ft-c and 0.015 M; (5) darkness and 0.15 M; (6) 600 ft-c and no sucrose. PAL activity was assayed at various time intervals as indicated. Each point in this plot represents an average of triplicate samples.](image2)

**Inactivation of PAL.**

The decay of PAL activity in leaf disks maintained in sucrose and light for longer than 48 hr suggests that inactivation as well as synthesis of enzyme occurred (Figs. 2 and 3). After 34 hr of incubation, transferring leaf disks to darkness caused rapid decay of PAL activity (Fig. 4, line 3). When the disks were transferred to darkness and to culture medium containing 20 µg/ml of cycloheximide, the rate of PAL decay decreased but cycloheximide did not stop the inactivation completely (Fig. 4, line 4), suggesting that an inactivating system was present in the disks before the transfer. Figure 4 (line 1) shows that more PAL activity was accumulated after further incubation of the leaf disks in sucrose and light. Addition of cycloheximide to disks maintained continuously in sucrose and light inhibited the further increase in PAL activity (Fig. 4, line 2). Sucrose and light appears to protect PAL from decay; otherwise the rate of decay in light (Fig. 4, line 2) should be similar to the rate of decay in darkness (Fig. 4, line 4). The fact that PAL inactivation occurred when leaf disks were transferred to darkness and sucrose with cycloheximide (Fig. 4, line 4) or to light and water with cycloheximide (Fig. 5, line 2) indicates that the protection of PAL against inactivation requires both light and sucrose.

When leaf disks which have been incubated for 34 hr in sucrose and light were transferred to water, PAL activity showed further increase for about 5 more hr, probably until the accumulated sucrose was consumed, then decreased rapidly (Fig. 5, line 1). Addition of cycloheximide did not prevent the decay (Fig. 5, line 2). The removal of both sucrose and light caused more rapid PAL inactivation (Fig. 5, line 3). The presence of cycloheximide decreased the rate of inactivation but did not completely inhibit the decay (Fig. 5, line 4).

Since the addition of cycloheximide did not completely inhibit PAL decay, it is concluded that the inactivating system must be present before the transfer of leaf disks. It is of interest...
to determine whether the activity of inactivating system increases as the period of preincubation in sucrose and light lengthens.

After 46 hr of incubation in light and sucrose, leaf disks were transferred to darkness and sucrose (Fig. 6, line 3). Immediate and rapid PAL inactivation was observed. The presence of cycloheximide (Fig. 6, line 4) decreased the rate of decay but did not inhibit the decay completely, suggesting again the presence of inactivating system in leaf disks before the transfer. Continuous incubation of disks in sucrose and light after 46 hr (Fig. 6, line 1) showed a slight increase in PAL activity and then decay occurred. In this experiment, light and sucrose with cycloheximide did not prevent decay (Fig. 6, line 2) in contrast to the results shown in Figure 4 (line 2). Thus, it appears that as the preincubation period lengthens, the inactivating system activity increases, and under these circumstances, light and sucrose cannot completely protect PAL from decay.

Transferring leaf disks after 46 hr of preincubation to water and darkness again caused decline of PAL activity (Fig. 7, line 3). The addition of cycloheximide decreased the rate of, but did not abolish the decay of PAL activity (Fig. 7, lines 2 and 4).

Table I clearly summarizes that the rate of PAL decay is faster in leaf disks which were preincubated in sucrose and light for 46 hr than in those disks preincubated for 34 hr. The half-life of PAL activity is significantly shorter in all cases, except case 3, in leaf disks preincubated for 46 hr than in those preincubated for 34 hr (Table I). Thus, the activity of inactivating system does increase as the periods of preincubation in sucrose and light lengthen.
Effect of Light on Accumulation of PAL Activity Is Linked to Photosynthesis. As shown in Figure 3, light accelerated the rate of PAL synthesis in leaf disks cultured in sucrose solution. The light requirement is apparently linked to photosynthesis. Figure 8 (line 2) shows that 25 µM DCMU, a specific inhibitor of photosystem II (15, 17), completely blocked the effect of light. The amount of PAL activity observed in the presence of DCMU is equivalent to the amount obtained during incubation in darkness (see Fig. 3). After 36 hr of preincubation in 0.1 M sucrose and light, the addition of DCMU caused a rapid decay of PAL activity (Fig. 8, line 3). When DCMU and cycloheximide were added together, the rate of decay was slower (Fig. 8, line 4). It is clear that the addition of DCMU has an effect similar to transferring leaf disks from light to darkness (see Figs. 4 and 6). The similar rates of decay in the presence of cycloheximide and cycloheximide with DCMU (Fig. 8, lines 4 and 5), indicate DCMU does not inhibit the PAL inactivating system and also indicate the presence of an inactivating system in leaf disks after 36 hr of preincubation.

Table 1. Summary of Half-Life of PAL Activity after Transferring Disks to Various Conditions for Inactivation

Values of half-life were calculated from plots of Figures 4 to 7.

<table>
<thead>
<tr>
<th>Second Incubation Conditions after Transfer</th>
<th>Length of Initial Incubation</th>
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<tbody>
<tr>
<td></td>
<td>36 hr</td>
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<tr>
<td>1. Sucrose + light</td>
<td></td>
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<tr>
<td>2. Sucrose + light + cycloheximide</td>
<td></td>
</tr>
<tr>
<td>3. Sucrose + dark</td>
<td>18.3</td>
</tr>
<tr>
<td>4. Sucrose + dark + cycloheximide</td>
<td>67.3</td>
</tr>
<tr>
<td>5. H₂O + light</td>
<td>22.0</td>
</tr>
<tr>
<td>6. H₂O + light + cycloheximide</td>
<td>39.0</td>
</tr>
<tr>
<td>7. H₂O + dark</td>
<td>16.7</td>
</tr>
<tr>
<td>8. H₂O + dark + cycloheximide</td>
<td>74.0</td>
</tr>
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</table>

Fig. 7. Effect of cycloheximide and removal of light and sucrose on the decay of PAL activity after prolonged preincubation. Leaf disks were first cultured in 0.1 M sucrose and light (600 ft-c) for 46 hr to induce accumulation of PAL activity. The disks were then washed thoroughly in water and transferred to various conditions: (1) H₂O and light; (2) H₂O and light and 20 µg/ml cycloheximide; (3) H₂O and darkness; and (4) H₂O and darkness and 20 µg/ml cycloheximide. PAL activity was assayed at intervals as indicated. Each point in this plot is an average of nine samples.

Fig. 8. Effect of DCMU on accumulation and inactivation of PAL activity. Leaf disks were maintained in 0.1 M sucrose and light (600 ft-c). At time indicated, the disks were transferred to light and sucrose (0.1 M) with the following substances added: (1) none; (2) 25 µM DCMU; (3) 25 µM DCMU and 20 µg/ml cycloheximide; and (5) 20 µg/ml cycloheximide. Another sample of disks was cultured continuously in 0.1 M sucrose and 25 µM DCMU in light (2). PAL activity was assayed at intervals as indicated. Each point in the plot is an average value of triplicate samples.

Fig. 9. Action spectrum of accumulation of PAL activity. Leaf disks were incubated in 0.1 M sucrose and light of different wavelengths as indicated. After 33 hr of incubation, PAL activity was assayed. The activity is plotted as a function of the wavelength of light used for each incubation. Each point in this plot represents an average of triplicate samples.

The relation between photosynthesis and effect of light on PAL accumulation was further demonstrated by action spectrum. Leaf disks cultured in 0.1 M sucrose and in light of different wavelengths showed two distinct peaks of PAL activity (Fig. 9), a lower peak at 475 nm and a higher peak at 625 nm. No PAL activity was detected at UV region (350 nm) or at far red region (740 nm) in contrast to the phytochrome controlled induction (8). The action spectrum of PAL accumulation is very similar to the action spectrum of photosynthesis (11). Thus, the chlorophylls instead of phytochromes appear to be the photoreceptors in this system.
of the PAL active site. Since it is stable, it could accumulate and inactivate PAL at an accelerating rate until it can inactivate PAL faster than PAL is synthesized, which leads to the rapid decline of PAL activity as shown in Figure 2. When the syntheses of PAL and PAL-inactivating system are inhibited in the leaf disks which have been preincubated in sucrose and light by the addition of cycloheximide, the accumulated inactivating system in the disks could continuously degrade PAL. Since the inactivating system is stable, it is reasonable to assume that more inactivating system is accumulated as the preincubation period lengthens. Figures 4 to 7 demonstrate that after addition of cycloheximide, the rate of inactivation of PAL is faster in leaf disks that have been preincubated for 46 hr than in those that have been preincubated only for 34 hr.

The requirement for external carbohydrate in PAL induction in strawberry leaf disks was reported earlier by Creasy (5, 6). The function of carbohydrate in the induction is, however, not understood. The levels of PAL in leaf disks are determined by the concentrations of sucrose (Fig. 2). Metabolism of exogenous carbohydrate could provide a source of ATP for the synthesis of PAL.

The requirement for light in PAL induction is also not clear, although it is apparently linked to photosynthesis (Figs. 8–10). High intensity light can remove the requirement of sucrose in strawberry leaf disks (6), presumably the higher rate of photosynthesis produces enough sucrose for PAL induction. However, high concentrations of sucrose cannot substitute for the requirement of light which indicates the function of light is not solely to supply carbohydrate. Light could facilitate the uptake of external carbohydrate as shown by Creasy (6) and Smith in Nitella (16). The light-stimulated glucose uptake by Nitella was reported to be DCMU-sensitive (16). Leaf disks incubated in high light intensity could have more rapid sucrose uptake which causes accelerated rate of PAL synthesis (Fig. 3). Another possible role of light is to provide a photosynthetic product which is required for the synthesis of PAL or to protect PAL against inactivation.

In darkness but in the presence of sucrose, a low level of PAL activity was detected (Fig. 3). This observation suggests that there could be two pools or two isozymes of PAL. The synthesis of one pool or one isozyme of PAL may require injury, sucrose, and light. The synthesis of another pool or another isozyme may require only injury and sucrose.

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LITERATURE CITED