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Temporal and Hormonal Control of β-1,3-Glucanase in *Phaseolus vulgaris* L.

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ABSTRACT

The endo-β-1,3-glucanase (β-1,3-glucan 3-glucanhydrolase, EC 3.2.1.6) extracted from *Phaseolus vulgaris* L. cv. Red Kidney had a pH optimum of 5 and a temperature optimum of 50 C. Excision of plant tissue resulted in an increase in β-1,3-glucanase activity after a 6-hour lag period. The increase could be prevented by indole-3-acetic acid, gibberellic acid, and cytokinins. Ethylene (half-maximal concentration = 0.1 microliter/liter) promoted the synthesis of β-1,3-glucanase, and 10% CO_2 overcame some of the ethylene effect. Cycloheximide prevented the induction of β-1,3-glucanase, but actinomycin D and chromomycin A_3 had only a partial effect.

The amount of callose in sieve tube cells correlated with levels of β-1,3-glucanase, suggesting that this enzyme played a role in the degradation of β-1,3-glucans.

Regulation of enzymatic activity is frequently incorporated into explanations of hormonal action on plants. Two examples include the increased digestion of starch by gibberellin-treated barley seeds (20) and cellulase induction in ethylene-regulated abscission (2). This paper presents data in favor of the view that regulation of another carbohydrase, β-1,3-glucanase, is important in the degradation of sieve tube callose and that a variety of plant hormones control the production of this enzyme.

MATERIALS AND METHODS

Plant Material. Bean (*Phaseolus vulgaris* L. cv. Red Kidney) plants were grown at 24 ± 2 C, 64% relative humidity, and a 12-hr photoperiod supplied by fluorescent and incandescent lamps that yielded 1200 ft-c at plant level. Petiole abscission zone explants (4.5-mm pulvinus, 5.5-mm petiole) from the primary leaf were excised from 2-week-old plants and inserted into a 3-mm layer of 1.5% agar contained in either 15-cm Petri plates or 43 ± 2 ml gas collection bottles. The gas collection bottles were 5 cm in diameter and 2.5 cm high and had a neck that accommodated a 2.5-cm diameter rubber vaccine cap. Except for experiments with RNA and protein synthesis inhibitors, the explants were treated by incorporating various chemicals in the agar. During the experiments, the explants were exposed to 25 C and 400 ft-c of continuous fluorescent light. The light, however, had no effect on induction of β-1,3-glucanase since results similar to those reported here were obtained with explants stored in the dark. The treatments were terminated by freezing the explants at −16 C and storing them at that temperature until subsequent extraction of enzyme. When whole plants were treated with ethylene, they were placed in plastic boxes (60 × 60 × 60 cm) located in a 25 C growth chamber with a 400 ft-c 16-hr photoperiod supplied by fluorescent lamps. Experimental data shown in the figures and tables are the means of two or three replicates and are representative of three experiments performed on separate occasions.

Reagents. The following chemicals were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio: indole-3-acetic acid, 75% K salt gibberellic acid, *N*-6-benzyladenine, actinomycin D, and laminarin. Zeatin was purchased from CalBiochem Corp., Los Angeles, California. Cytokinin SD 8339 6-benzylamino-9,2-(tetrahydropyranyl-9 H purine) was a gift of the Shell Development Co., Modesto, California. The R. J. Reynolds Tobacco Co., Winston-Salem, North Carolina, kindly supplied the abscisic acid (47.3% *cis,trans*). The pachyman used to make carboxymethylpachyman was a gift of Dr. B. A. Stone, University of Melbourne, Parkville, Victoria, Australia. The carboxymethylpachyman was made according to the method described by Clarke and Stone (6). The procedure was modified by adding 3 liters of 2-propanol to the original reaction mixture of pachyman and NaOH. Chromomycin A_3 was purchased from the Mann Research Laboratories, New York, New York.

Explants were treated with ethylene and CO_2 by adding appropriate dilutions of these gases to the gas phase inside the gas collection bottles with a syringe inserted through the rubber vaccine cap. A flame ionization gas chromatograph fitted with a ¼-inch, 60-cm activated alumina column run at 100 C was used to measure ethylene.

Actinomycin D (1 μg), cycloheximide (0.25 μg), and chromomycin A_3 (5 μg) were injected as 1-μl solutions into bean explants with a microliter syringe by inserting the needle up through the center of the petiole tissue to a depth of about 5 mm, at which point the firmer pulvinal tissue resists further movement of the needle.

β-1,3-Glucanase (β-1,3-glucan 3-glucanohydrolase, EC 3.2.1.6) was extracted from plant tissue by homogenizing 10 explants, or an equivalent amount of other tissue, in 4 ml of 0.05 M, pH 5, potassium acetate buffer in a Ten Broeck homogenizer. The homogenates were filtered through Mira-cloth (CalBiochem Corp.) premoistened with acetate buffer, centrifuged at 10,000 *g* for 10 min, and the resulting supernatant liquid was retained for subsequent assay of activity. The β-1,3-glucanase is a soluble enzyme since centrifugation at 100,000 *g* for 1 hr removed only 16% of the original activity from the crude homogenate and no activity could be recovered from the precipitate.

Both carboxymethylpachyman and laminarin were used as substrates for the β-1,3-glucanase. The reaction of β-1,3-glucanase with carboxymethylpachyman was measured in a Wells-Brookfield microviscometer (Brookfield Engineering Laboratories, Stoughton, Massachusetts). Equal volumes of enzyme and 2% carboxymethylpachyman were mixed together, and a 1-ml sample was placed in the microviscometer. The resulting viscosity after a 10-min incubation at 40 C (timed from the moment of mixing) indicated the activity of the enzyme. The viscometric technique was a sensitive method of measuring β-1,3-glucanase, but it was

time-consuming and not suited to measuring large numbers of samples. For routine experiments, a colorimetric assay utilizing laminarin as a substrate and the dinitrosalicyclic reagent (prepared by adding 300 ml of 4.5% NaOH to 880 ml of a solution containing 8.8 g of dinitrosalicylic acid and 255 g of potassium sodium tartrate $6H_2O$) was used to measure the reducing sugars produced. After initial experiments revealed that optimum conditions for assay were pH 5 and 50 C, 0.5 ml of enzyme in 0.05 M, pH 5, potassium acetate buffer was routinely added to 0.5 ml of 2% (w/v) laminarin in water and incubated at 50 C for 1 or 2 hr. The laminarin was dissolved by heating the 2% solution briefly in a boiling water bath before use. The reaction was stopped by adding 3 ml of the dinitrosalicylic reagent and heating the tubes for 5 min at 100 C. The tubes were then cooled to 25 C, the contents were diluted 1:10 with water, and the optical density was read at 500 nm. Figure 1 presents data comparing the viscometric and colorimetric assays for β-1,3-glucanase. The Nelson reagent was also utilized in a number of experiments and gave similar results (15). Because of the complex nature of the reaction, straight line kinetics were not obtained with these assays. However, at lower enzyme concentrations, the change in optical density and viscosity gave a practical approximation of relative enzyme activity. Lack of linearity was due in part to the fact that the β-1,3-glucanase is an endo- as opposed to exo-enzyme. This was established by chromatographing the reaction products on a thin layer of cellulose with isopropanol-acetic acid-water (67:10:23). To reveal the sugars produced, the chromatograms were sprayed with benzidine (0.5 g of benzidine, 20 ml of glacial acetic acid, 80 ml of absolute ethyl alcohol) and heated at 100 C for 5 min. The β-1,3-glucanase released no observable glucose from the laminarin, but rather a series of products (presumably β-1,3-oligosaccharides) that chromatographed behind a glucose standard. The failure of the glucose oxidase reaction (5) to give a positive result with the products of a β-1,3-glucanase digestion supported chromatographic evidence.

Callose deposits were measured according to the methods of Scott *et al.* (17).

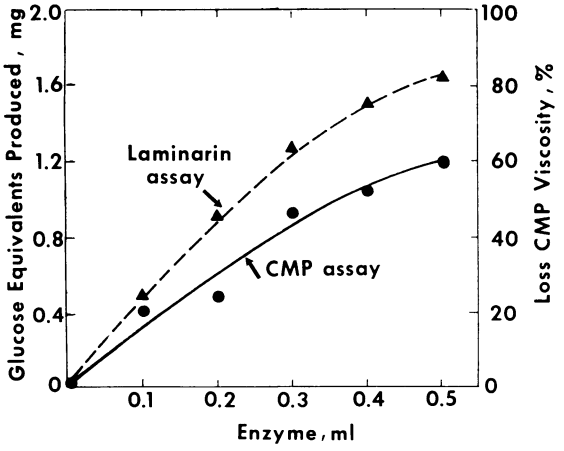

FIG. 1. Comparison between colorimetric and viscometric assays for β-1,3-glucanase. For the laminarin assay 0.5 ml of 2% laminarin was added to 0.5 ml of an enzyme buffer (0.05 M, pH 5, acetate) mixture and incubated at 50 C for 2 hr. Reducing groups were measured by the dinitrosalicylic method. The viscometric assay utilized a 1-ml sample containing 0.5 ml of 2% carboxymethylpachyman (CMP) and 0.5 ml of enzyme acetate buffer mixture. The activity of the enzyme was expressed as the percentage loss of viscosity of the mixture after a 10-min 40 C incubation period compared with a substrate buffer control.

RESULTS

Localization of β-1,3-Glucanase Activity in the Bean Plant. All of the above-ground portions of the bean plant contained β-1,3-glucanase, and the activity of the enzyme increased in aged excised leaf and stem tissue. Enzyme activity also increased with age in attached leaves. Figure 2 shows representative data on the increase in β-1,3-glucanase activity in abscission zone explants with time as measured both viscometrically with carboxymethylpachyman as the substrate and colorimetrically with laminarin as the substrate. The difference in time required for the increase in activity between the two curves is due in part to the fact that the experiments were performed at different times. After these initial experiments, the viscometric assay, though more sensitive, was discontinued because more samples could be examined colorimetrically in an equivalent amount of time.

The increase in β-1,3-glucanase activity occurs for at least 3 days, as shown in Figure 3. When the experiments were originally

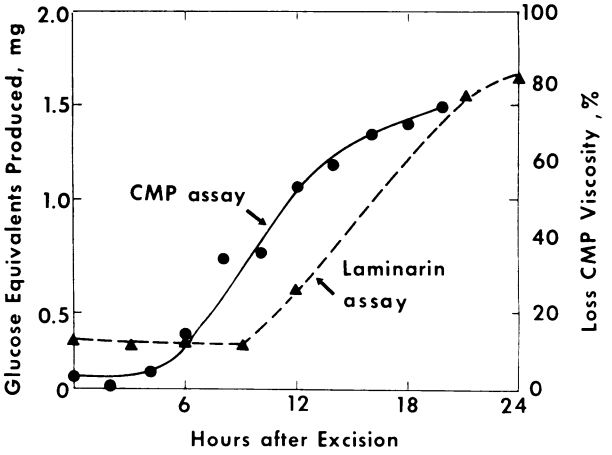

FIG. 2. Increase in β-1,3-glucanase activity in abscission zone explants measured colorimetrically and viscometrically. Explants were excised and stored on plain agar in Petri plates and frozen at the times indicated. CMP: Carboxymethylpachyman.

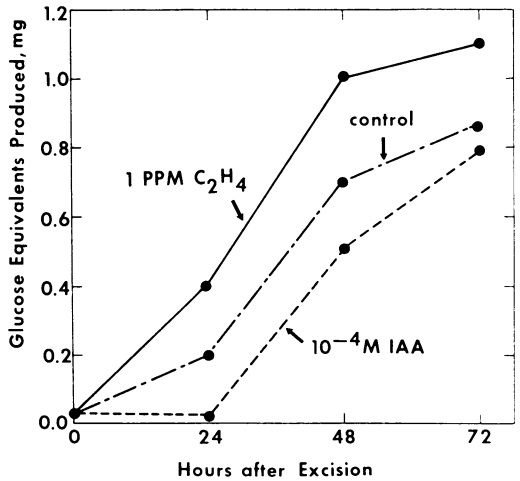

FIG. 3. Time course for the induction of β-1,3-glucanase activity in abscission zone explants in the presence of 1 μl/liter ethylene and 10^{-4} M IAA. The explants were placed in gas collection bottles containing plain agar or 10^{-4} M IAA. The explants were placed pulvinal end-down in the IAA to facilitate transport into the tissue. Explants were harvested at the time indicated and frozen until subsequent extraction of β-1,3-glucanase. Flasks were vented and resealed at 24 and 48 hr and ethylene was reinjected at those times. Because of the high activity after 3 days, only 0.1 ml of enzyme was used in the laminarin assay.

planned, it was felt that β-1,3-glucanase might play some role in abscission, and so both abscission-accelerating (ethylene) and inhibitory (IAA) hormones were applied to the explants. The data indicate that ethylene increased β-1,3-glucanase activity but IAA delayed the increase. Transferring IAA-treated explants to fresh IAA after 1 day had no further effect on blocking the increase.

The idea that the increase in β-1,3-glucanase represented a wounding response was tested by subdividing 10 1-cm petiole sections into two, three, and four pieces and comparing their β-1,3-glucanase activity with that of intact sections after a 24-hr aging period. It was found that subdividing the petiole sections caused a loss rather than increase in β-1,3-glucanase activity. The wounding explanation was tested further by harvesting explants a day after they had been scratched with a needle and comparing them with untreated controls. No difference was found between explants from treated and control plants. It was also possible that the increase in activity was due to changes in inhibitors and activators in the tissue. To check this explanation, extracts from initial and aged explants were deproteinized by heating them to 100 C for 10 min and then adding samples back to enzyme solutions from initial or aged explants. These boiled solutions had no effect on enzyme activity from initial or aged explants and had no activity themselves.

Effect of Ethylene. A concentration curve is shown in Figure 4 and, characteristic of ethylene effects, 0.1 μl/liter was approximately half-maximal and 10 μl/liter was essentially saturating. The characteristic reversal of ethylene action by CO_2 is shown in Table I. However, the effect was small and 10% CO_2 was unable to reverse completely the effect of 1 μl/liter ethylene or reduce the control value to the initial activity. The maximum rate of increase of β-1,3-glucanase activity depended on a continual supply of ethylene. Removal of ethylene after 14 hr caused the rate of increase to slow to that shown by controls.

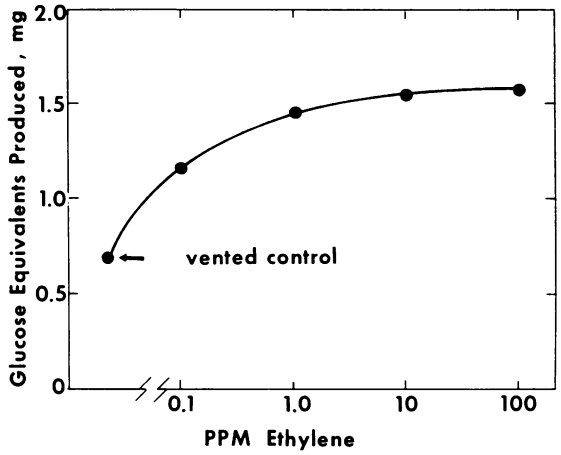

FIG. 4. Increase in β-1,3-glucanase activity by ethylene. Explants were treated with ethylene in gas collection bottles. The bottles were vented and resealed after an initial 7 hr to remove wound ethylene. Vented control bottles were covered with cheesecloth instead of the usual rubber vaccine stopper. Explants were frozen 24 hr after the start of the experiment and stored until subsequent extraction for β-1,3-glucanase activity.

Effect of Other Hormones. Figure 5 shows that IAA, GA, and SD 8339 were capable of inhibiting the increase of β-1,3-glucanase activity. Table II presents data which show that, of the cytokinins available for testing, SD 8339 was the most effective in blocking the increase in β-1,3-glucanase activity. The action of these hormones was complex. Table III presents data on the effect of low concentrations of these substances added singly or in combination to explants. When IAA, GA, and SD 8339 were added in combination, they had a synergistic effect compared with the action of these hormones alone. Abscisic acid was also tested for its effect on the increase in β-1,3-glucanase activity. However, concentrations up to 10^{-4} M were inactive.

Figure 6 shows the effect of adding IAA at 2-hr intervals after excision of the explants. The data show that IAA was capable of blocking the increase in β-1,3-glucanase activity when added up to 6 hr after excision of the explants.

Effect of RNA and Protein Synthesis Inhibitors. Table IV shows that cycloheximide blocked the increase in β-1,3-glucanase activity but actinomycin D had only a partial effect. Another RNA

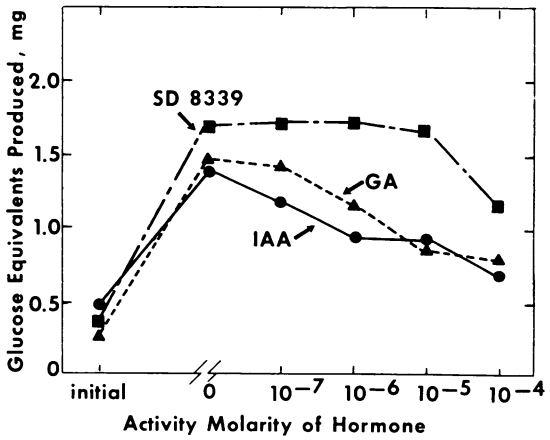

FIG. 5. Inhibition of β-1,3-glucanase activity by plant hormones. Explants were inserted pulvinal end-down in Petri plates containing the substances to be tested. Except for initials, explants were harvested after 24 hr of treatment and frozen until subsequent determination of β-1,3-glucanase activity.

Table I. *Increase of β-1,3-Glucanase Activity by 1 μl/liter Ethylene and Reversal by 10% CO_2*

Explants placed petiole end-down in 1.5% agar in sealed bottles for 24 hr in the gas phases indicated. Bottles were vented and resealed, and ethylene or CO_2 or both were reinjected 6 hr after the start of the experiment.

Treatment	Glucose Equivalents Produced
	mg
0 hr initial	0.49
Control	1.20
Ethylene	1.73
CO_2	1.11
Ethylene + CO_2	1.51

Table II. *Inhibition of β-1,3-Glucanase Induction by the Cytokinins, N-6-Benzyladenine, SD 8339, Zeatin, and Kinetin*

Explants were placed pulvinal end-down in 1.5% agar containing the cytokinins indicated at a concentration of 10^{-4} M for 24 hr before determination of enzyme activity.

Treatment	Glucose Equivalents Produced
	mg
0 hr initial	0.34
Control	1.07
SD 8339	0.47
Kinetin	0.68
N-6-Benzyladenine	0.70
Zeatin	0.73

synthesis inhibitor, chromomycin A_3, which has an action similar to that of actinomycin D (12, 14), was also tested and had an effect similar to that of actinomycin D. The amounts of inhibitor used represent the minimal concentration that would completely block abscission of the explants. Since both RNA and protein synthesis are required for abscission (1), this served as a check for the effectiveness of these compounds on RNA and protein synthesis.

Table III. *Inhibition of Induction of β-1,3-Glucanase Activity by IAA, GA, and Cytokinin SD 8339*

Explants were placed pulvinal end-down in 1.5% agar containing 10^{-5} M IAA, 10^{-5} M GA, or 10^{-4} M SD 8339 singly or in combination as indicated, for 24 hr before determination of enzyme activity.

Treatment	Glucose Equivalents Produced
	mg
0 hr initial	0.55
Control	1.27
IAA	1.23
GA	1.00
SD 8339	0.68
IAA + GA	0.62
IAA + SD 8339	0.47
GA + SD 8339	0.62
IAA + GA + SD 8339	0.33

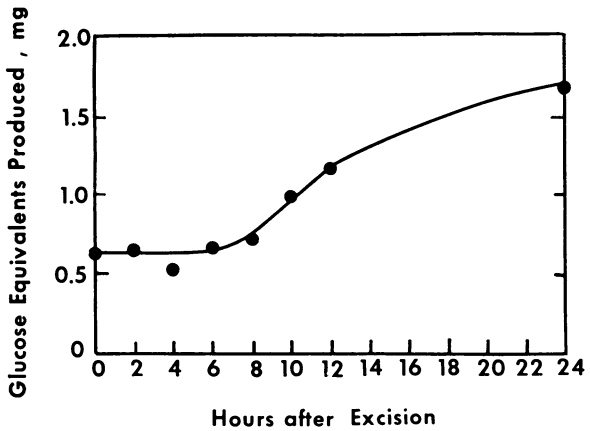

FIG. 6. Time course for the inhibition of β-1,3-glucanase by 10^{-4} M IAA. Explants were excised and placed in Petri plates containing plain agar. At the time indicated they were inserted pulvinal end-down in IAA agar. The explants were harvested 24 hr after the start of the experiment and frozen.

Table IV. *Inhibition of Ethylene-induced β-1,3-Glucanase Activity by Cycloheximide and Actinomycin D*

Explants were injected with 1 μl of cycloheximide (0.25 μg/μl) or actinomycin D (1 μg/μl) after excision and inserted petiole end-down in bottles containing 1.5% agar. Bottles were sealed, and, as required, 1 μl/liter ethylene was added to the gas phase.

Treatment	Glucose Equivalents Produced		
	Cyclo-heximide	Actinomycin D	Chromo-mycin A_3
	mg		
0 hr initial	0.22	0.14	0.22
Control	1.22	1.59	0.92
Ethylene	2.01	1.67	1.77
Inhibitor	0.24	1.54	0.81
Ethylene + inhibitor	0.20	1.57	0.90

Cycloheximide was also used to show that the β-1,3-glucanase was stable in the tissue once it was formed. In these experiments day-old explants were injected with cycloheximide, and enzyme activity was measured 4, 8 and 24 hr later. We found that during the 24-hr period following injection β-1,3-glucanase remained constant and equal to the level reached prior to the cycloheximide injection. Figure 7 shows that cycloheximide can be injected into explants as much as 7 hr after they are excised from the plant and still block most of the subsequent increase in β-1,3-glucanase activity.

Sieve tube callose is a carbohydrate containing β-1,3-linkages and as such represents a substrate for increased levels of β-1,3-glucanase. Table V shows that β-1,3-glucanase activity in pulvinal tissue of explants correlated inversely with the number of callose deposits in the same tissue. In another experiment (Table VI) whole plants were gassed with 10 μl/liter ethylene and were found to produce large quantities of β-1,3-glucanase in the leaves. Callose deposits in the pulvini of these leaves decreased during this same period. After 3 days of ethylene fumigation the plants were completely defoliated, and the leaves were wilted and chlorotic.

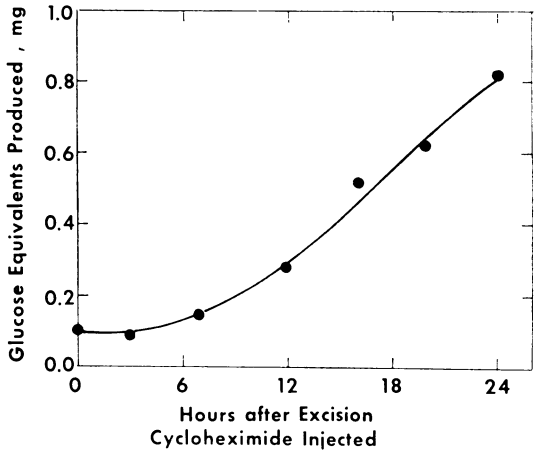

FIG. 7. Time course for the inhibition of β-1,3-glucanase by 0.25 μg of cycloheximide. Explants were excised and stored on plain agar. At the times indicated they were injected with 1 μl of cycloheximide solution and returned to the agar. After 24 hr of incubation, the explants were harvested and frozen.

Table V. *Frequency of Sieve Tube Callose Deposits in the Pulvinus of Bean Leaf Abscission Zone Explants after Aging in the Presence of Various Chemicals*

Longitudinal sections 32 μ thick were prepared from 4 pulvini and stained with 0.005% aniline blue in 0.15 M, pH 8, potassium phosphate buffer. The number of fluorescent sieve tube end walls observed in 10 0.62-mm diameter fields from each pulvinus was counted and averaged. β-1,3-Glucanase activity represents 0.25 ml of enzyme preparation extracted from 10 pulvini.

Treatment	No. of Callose Deposits	β-1,3-Glucanase
		mg glucose equivalents
Initial	41 ± 4	0.54
24 hr control	11 ± 6	1.18
24 hr 10^{-4} M IAA	12 ± 3	0.81
24 hr 10 μl/liter ethylene	9 ± 5	1.89
24 hr 0.25 μg cycloheximide	33 ± 6	0.59

Table VI. *Effect of 10 μl/liter Ethylene on Callose and β-1,3-Glucanase from Bean Leaves*

Longitudinal sections 32 μ thick were prepared from 4 pulvini and stained with 0.005% aniline blue in 0.15 M, pH 8, phosphate buffer. The number of fluorescent sieve tube end walls observed in 10 0.62-mm diameter fields from each pulvinus was counted and averaged. β-1,3-Glucanase was assayed by extracting leaf blade tissue with 0.05 M, pH 5, acetate buffer (1 ml of buffer per g of tissue) and incubating 0.1 ml of homogenate after 10,000 *g* × 10 min centrifugation with 10 mg of laminarin in 0.9 ml of 0.05 M, pH 5, acetate buffer at 50 C for 1 hr.

Day	No. of Callose Deposits		β-1,3-Glucanase	
	Control	Ethylene	Control	Ethylene
			mg glucose equivalents	
0	62 ± 12	...	0.0	...
1	58 ± 12	39 ± 20	0.02	0.23
2	60 ± 14	36 ± 13	0.03	0.46
3	64 ± 15	31 ± 15	0.02	0.62

DISCUSSION

The biochemical properties of bean β-1,3-glucanase reported here (pH optimum of 5, 50 C optimum, and nonparticulate nature) are similar to those reported by others studying plant β-1,3-glucanase (6, 10, 13). However, Clarke and Stone (6) reported that the β-1,3-glucanase preparation they isolated from grape vines produced glucose from laminarin while the bean enzyme was an endoenzyme producing only oligosaccharides from laminarin. The β-1,3-glucanase studied by Mandels *et al.* (13) and Esser (10) were also endoenzymes.

The observations that 0.1 μl/liter ethylene was half-maximal in increasing β-1,3-glucanase (Fig. 4) and that CO_2 was partially able to overcome the ethylene effect (Table I) are typical of ethylene action in plants. An earlier paper from this laboratory reported analogous results with the effect of ethylene on abscission and summarized similar examples from earlier literature on other ethylene-mediated responses (3). Apparently the attachment site of ethylene in the cell that eventually gives rise to an increase in β-1,3-glucanase activity is the same or similar to the one that regulates abscission (RNA-dependent cellulase synthesis), ripening, inhibition of cell elongation, peroxidase synthesis, and flower fading. Cytokinins, auxins, and gibberellins delayed the appearance of β-1,3-glucanase activity in excised bean tissue (Figs. 5 and 6 and Tables II and III). Why these basically dissimilar compounds have corresponding effects is puzzling. It should be noted, however, that the concentrations used to block β-1,3-glucanase synthesis are high compared with those for the normal growth regulator effects ascribed to these hormones in plants. It is possible, however, that the aging- or senescence-retarding capabilities these materials display at high concentrations may be a part of the explanation. In other words, excision of plant tissue sets off an aging phenomenon as the endogenous supply of aging retardants are used up in the tissue and no replenishment from the normal source of supply is possible. The effect is demonstrated in Figure 6. The data indicate that IAA can be added to the explant 6 hr after it has been excised and still block the subsequent rise of β-1,3-glucanase activity. This is analogous to what has been observed with the induction of cellulase (a β-1,4-glucanase) during abscission. IAA can be added to abscission zone explants up to 6 hr after excision and still block the formation of cellulase (7).

It appears likely that inhibition of β-1,3-glucanase by these plant hormones occurs in different ways. If IAA, GA, and SD 8339 were having exactly the same effect, then combinations of these hormones should have additive effects. However, the data in Table III suggest an interaction of the hormones because a synergistic effect was observed. The effect of IAA on β-1,3-glucanase has been studied earlier by others. Unlike the results presented here, they found that auxin either promoted (19) or had no effect on β-1,3-glucanase.

The ability of cycloheximide to block the appearance of β-1,3-glucanase activity is taken as evidence in favor of the idea that the increased β-1,3-glucanase activity is due to protein synthesis *de novo* (Table IV). However, positive proof will ultimately depend on the demonstration that radioactively labeled amino acids are incorporated into a purified enzyme. The β-1,3-glucanase induction curve shown in Figure 2 indicates that about 6 hr are required after excision before the enzyme is synthesized. This was demonstrated another way by showing that an injection of cycloheximide lost its full inhibitory effect by 6 hr after excision (Fig. 7).

On the other hand, inhibitors of RNA synthesis (actinomycin D, chromomycin A_3) had only a partial effect on β-1,3-glucanase induction. Lack of effectiveness is not thought to be due to inactivity of these compounds because they completely blocked abscission in this tissue, which we had shown earlier to require RNA synthesis (11). The data in Table IV suggest either that the temporal effect does not need RNA synthesis and the ethylene effect does, or that both processes have some requirement blocked by these antibiotics.

A number of functions for β-1,3-glucanase in plant tissue have been suggested. These include degradation of seed glucans (9), control of cell elongation (18), regulation of pollen tube growth (16), cell expansion of yeast (18), fertilization (10), and removal of callose (6).

Data in Tables V and VI present evidence in favor of the last idea. Earlier, Scott *et al.* (17) reported that ethylene-treated bean abscission zone explants had less callose than controls and that auxin-treated ones had more. Using their methods and measuring the fluorescence of phloem stained with aniline blue, we found that callose deposition correlated with β-1,3-glucanase activity. Such data are consistent with, but do not prove, the suggestion that one function of β-1,3-glucanase is to regulate the amount of β-1,3-glucans plugging sieve tube cell plates. Alteration of callose in phloem may explain the observation that ethylene stimulates exudation of liquids from lenticels (4). On the other hand, it is unlikely that β-1,3-glucanase plays any role in abscission. Unlike cellulase, β-1,3-glucanase activity is not localized in the separation layer. Secondly, even though actinomycin D and chromomycin A_3 block abscission, they do not completely prevent the increase in β-1,3-glucanase. Finally, even though gibberellic acid was able to block the temporal increase in β-1,3-glucanase, it does not prevent abscission.

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