

Abscission: Role of Cellulase

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Abstract. Cellulase (β -1,4-glucan-glucanohydrolase EC 3.2.1.4) activity increased during abscission and was localized in the cell separation layer of *Phaseolus vulgaris* L. cv. Red Kidney (bean), *Gossypium hirsutum* L. cv. Acala 4-42 (Cotton) and *Coleus blumei* Benth. Princeton strain (*Coleus*) abscission zone explants. Cellulase activity was optimum at pH 7, was reduced by one-half after heating to 55° for 10 min, and was associated with the soluble components of the cell. Explants treated with aging retardants (indoleacetic acid, ⁶N-benzyladenine, and coumarin), CO₂, actinomycin D or cycloheximide had less cellulase activity than untreated controls. Ethylene increased cellulase activity of aged explants after a 3-hr lag period but had no effect on cellulase activity of freshly excised explants. It was concluded that 1 of the roles of ethylene in abscission is to regulate the production of cellulase which in turn is required for cell separation.

A number of investigators have explored the role of cell-wall degrading enzymes in abscission. Osborne (19) reported that high levels of pectin methyl-esterase were associated with the separation layers of *Phaseolus vulgaris* L. (bean) abscission zone explants in which abscission was delayed by treatment with 2,4-dichlorophenoxyacetic acid. Acceleration of abscission by ethylene resulted in reduced levels of pectin methyl-esterase. Yager (24) reported similar results with *Nicotiana tabacum* L. (tobacco) explants. In his experiments indoleacetic acid was used as the abscission retardant and methionine as the accelerant. However, it is likely that the stimulatory effect of methionine was due to a stimulation of ethylene production (1). Rasmussen (20) also reported that decreasing levels of pectin methyl-esterase were associated with aging bean explants. Rasmussen also found that polygalacturonase activity decreased during abscission.

Using the loss of cellular material from cucumber slices as an assay for pectinase, Morre (17) reported that increasing enzyme activity was associated with abscission of bean petiole explants. A second enzyme known to increase during abscission is cellulase. Horton and Osborne (14) reported that cellulase activity was localized in the separation layer of bean explants and that 2,4,5-trichlorophenoxyacetic acid inhibited cellulase activity while ethylene increased it.

Ethylene action during abscission is thought to be hormonal; that is, regulating the production of enzymes required for cell separation by regulating RNA and protein synthesis (2). This report presents experiments designed to support the above hypothesis, by showing that the regulation of cellulase activity by ethylene corresponds to data obtained earlier on the regulation of RNA and protein synthesis by ethylene.

Materials and Methods

Cellulase (β -1,4-glucan-glucanohydrolase EC 3.2.1.4) was assayed by measuring the loss of viscosity of a sodium carboxymethyl cellulose (CMC) solution with a model LVT Wells-Brookfield microviscometer (Brookfield Engineering Laboratories, Stoughton, Massachusetts). This cone plate viscometer was found to be especially useful for measuring small samples (1 ml) of cellulase (21) over a range of 0 to 2000 centipoises and has been described earlier (23). The cellulase activity reported in this paper represents C_x cellulase as opposed to C₁ cellulase according to the criteria of Mandels and Reese (16). C₁ cellulase acts on native or crystalline cellulose in such a way that subsequent action by C_x cellulase becomes possible. C_x is thought to act mainly on modified cellulose or cellulose derivatives such as CMC. Operationally, C₁ cellulase activity is measured by its action on absorbent cotton, using an increase in reducing groups as the criterion of activity. However, explant cellulase failed to hydrolyze absorbent cotton even after prolonged incubation and at a variety of pH's.

A 1.5% CMC solution was prepared by slowly adding powdered CMC (Nutritional Biochemicals Corporation) to 0.05 M potassium phosphate, pH 7, 0.05 M NaF in a Waring Blendor and then autoclaving the mixture for 15 min. After the solution cooled, toluene to give a 0.5% solution was added as a preservative.

Cellulase activity was measured by adding 1 ml of enzyme solution to 1 ml of 1.5% CMC held at 40°. Enzyme preparations with high activity were analyzed by placing 1 ml of the enzyme substrate mixture in the viscometer and determining the viscosity 10 min after mixing. The viscosities of prep-

arations with lower activity were determined after a 4- to 6-hr incubation period at 40°. The enzyme substrate mixture was equilibrated in the viscometer for 10 min before determining viscosity. While methods for expressing cellulase activity in absolute terms have been proposed (8,9), it is simpler and just as meaningful to present data as the percent change in viscosity of the CMC plus enzyme solution compared to a blank without enzyme (% $\Delta\eta$). The viscosity of a 0.75% CMC solution was about 50 centipoises.

Methods for growing, preparing, and storing *Phaseolus vulgaris* L. var. Red Kidney (bean), *Gossypium hirsutum* L. cv. Acala 4-42 (cotton), and *Coleus blumei* Benth. Princeton strain (coleus) explants were described earlier (5). For most of the experiments described in this paper, bean explants were placed petiole-end down and cotton and coleus explants stem-end down in a 3-mm deep layer of 1.5% agar in petri plates. Bean explants treated with indoleacetic acid (IAA), coumarin, or the cytokinin ⁶N-benzyladenine were placed pulvinal-end down in the agar. Actinomycin D (1 μ g) and cycloheximide (0.25 μ g) were injected as 1 μ l solutions into bean explants with a microliter syringe by sticking 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. A water injection was used as a control in these experiments and had no effect on abscission.

Except for CO₂ experiments, explants were treated with ethylene by placing the petri plates in 10-liter desiccators. The contents of the desiccators were first subjected to a partial vacuum, and then ethylene and CO₂ were added to the gas phase by means of a syringe inserted through the rubber vaccine cap covering the desiccator outlet. The vaccine cap was then removed to equilibrate the contents to atmospheric pressure.

For convenience, the methodology for each experiment is described with the presentation of results because of the variation of specific details among experiments.

Results

Preliminary experiments consisted of assaying for cellulase activity in various parts of abscission zone explants before and after abscission. In the case of bean explants, the pulvinus represents the top 3 mm, the separation layer the middle 2.5 mm, and the petiole the remaining 4.5 mm. The petiole of cotton explants represents the top 2.5 mm of cotyledonary petiole, the separation layer the remaining petiole tissue flush with the stem and nodal tissue the stem tissue between the 2 petiole bases. Node number 4 *Coleus* explants were sub-divided in a similar manner. To insure that the cellulase activity did not represent bacterial contamination, bean and cotton explants were surfaced sterilized with a 30-sec wash

of 2% NaOCl, followed by 2 rinses of sterile distilled water. The explants were then stored in sterile agar. Sterile homogenates of abscising explants were free of bacterial contamination when plated out on nutrient agar. Since essentially similar data on cellulase production were obtained from explants isolated under non-sterile conditions, the NaOCl treatment was not used in subsequent experiments.

Cellulase was extracted from bean and cotton explants by homogenization in 10 ml of 0.05 M potassium phosphate buffer, pH 7 in a VirTis homogenizer. *Coleus* sections were ground in a Ten Broeck homogenizer with 4 ml of buffer. Polyvinylpyrrolidone (2%) was added to the buffer in the case of cotton and *Coleus* explants to protect the enzyme from any possible detrimental effect of gossypol and other phenolic substances present in these tissues. The homogenates were filtered through Miracloth (Calbiochem Corporation) and centrifuged at 10,000g for 10 min. Cellulase activity of the supernatant fluid is indicated by the data in table I. Freshly excised explants were free of cellulase activity, but significant amounts of the enzyme were found in the abscising explants. In bean explants, cellulase activity was greatest in the separation layer, less in the pulvinus and absent in the petiole. In cotton and *Coleus* explants, cellulase was also localized in the separation layer with lesser amounts in the surrounding tissues.

Table I. Localization of Cellulase Activity in Abscission Zone Explants

Initial cellulase activity was determined on freshly excised explants (100 for bean, 65 for cotton, and 12 for *Coleus*) subdivided into petiole, separation layer, and nodal tissue. An equal number of explants were aged a day in air, a day in ethylene before being subdivided and analyzed for cellulase activity.

Explant section	% $\Delta\eta$ 10 Min	
	Initial activity	1 ppm C ₂ H ₄ treatment
	Bean ¹	
Pulvinus	+4	-20
Separation layer	+7	-46
Petiole	+1	+6
	Cotton ¹	
Petiole	-8	-2
Separation layer	-6	-20
Node	+2	-13
	<i>Coleus</i>	
Petiole	+4	-35
Separation layer	-2	-61
Node	+7	-41

¹ Surface sterilized with 2% NaOCl.

Most of the cellulase activity of bean explants appears to be soluble. To show this 250 bean explants were aged 24 hr in air, 24 hr in 10 ppm ethylene, homogenized with 10 ml 0.05 M phosphate buffer, pH 7, and then filtered through Miracloth. A sample of the filtered homogenate was assayed

Table II. Fractionation of Abscission Zone Cellulase by Centrifugation

Abscission zone explants (250) were aged a day in air then a day in 10 ppm ethylene before extraction with 10 ml phosphate, pH 7, buffer.

Fraction	% $\Delta\eta$ 10 Min	
	Precipitate	Supernatant
Original homogenate		46
2000g \times 10 min	13	46
100,000g \times 10 min	10	36
100,000g \times 1 hr	6	33

directly in the viscometer. The remaining homogenate was centrifuged at 2000g for 10 min and the resulting pellet resuspended in buffer so that the volume of resuspended precipitate and supernatant were equal. Samples of both the precipitate and supernatant were assayed for cellulase activity. This procedure was repeated at 10,000g for 10 min and 100,000g for 1 hr using the subsequent supernatant solutions. The data in table II indicate that the cellulase activity remained primarily in the supernatant fractions.

It was possible to precipitate cellulase with NH_4SO_4 and acetone. Supernatant (10,000g for 10 min) from aged ethylene treated explants was brought to 20% NH_4SO_4 , held at 0° for 15 min, and centrifuged at 10,000g for 10 min. The supernatant was then brought to 80% saturation, held at 0° for 30 min, and centrifuged at 10,000g for 10 min. The final pellet was then taken up in an amount of buffer equal to the volume of original solution of crude cellulase. The cellulase was also precipitated by adding 2 ml of 0° acetone for each ml of supernatant and centrifuging at 3000g for 10 min. After washing the precipitate twice with 70% acetone v/v with water), it was taken up in a volume of phosphate buffer equal to that of the original crude preparation. None of the cellulase activity was lost in the NH_4SO_4 fractionation, while about 80% of the initial activity was recovered after precipitation with acetone.

The effect of pH on cellulase activity was measured by homogenizing ethylene-treated explants in water and mixing portions of supernatant centrifuged at 10,000g for 10 min with equal volumes of MacIlvains buffer (0.1 M K_2HPO_4 + 0.05 M citric acid). The cellulase activity of these samples was measured on CMC dissolved in water. Cellulase activity was greatest at pH 7.0 with a smaller peak at pH 3.4. Subsequently, all extractions and analysis were performed with 0.05 M potassium phosphate buffer, pH 7.

Cellulase activity increased about 2-fold from 30° to 50°. It was not possible to obtain higher temperatures with the water bath used to supply water to the Wells-Brookfield viscometer.

The heat stability of cellulase was tested by exposing aliquots of the enzyme to different temperatures for 10 min and measuring subsequent cellu-

lase activity at 40°. A 10-min treatment at 40° had no effect on activity, a 55° treatment caused 50% loss in activity, while 60° inactivated the enzyme when compared to a control held at 0°.

A comparison between bean and commercial cellulase (Calbiochem Corporation) concentration and the reduction of CMC viscosity is shown in Fig. 1. This figure indicates that the viscometric technique described here was able to measure as little as 1 μg of cellulase after 10 min.

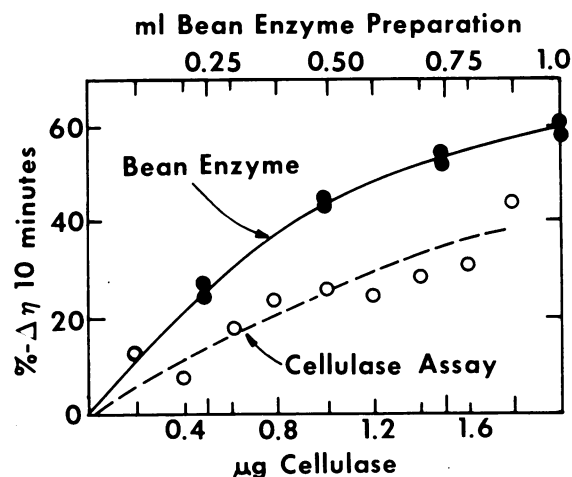


Fig. 1. Enzyme concentration curve. Effect of increasing concentrations of abscission-zone cellulase and commercial cellulase preparations on the viscosity of CMC. Explants (190) were aged in air for 24 hr, in 10 ppm ethylene 24 hr, and homogenized in 8 ml 0.05 M phosphate buffer, pH 7. The homogenate was filtered thru Miracloth and centrifuged at 10,000g for 10 min. Various portions of the homogenate were mixed with 1.5% CMC (phosphate buffer was used to make up the differences in volume) and the viscosity measured after 10 min. The cellulase was used as purchased from a commercial source.

The time course for cellulase formation in explants that received a 20-hr aging period is shown in Fig. 2. Aged explants were used in this and subsequent experiments because earlier work showed that freshly excised explants are insensitive to applied ethylene (7). As a check, freshly harvested explants were exposed to 10 ppm ethylene for 8 hr and the cellulase activity measured after a 6 hr incubation in CMC. The cellulase content from initial 8 hr controls, and 8 hr ethylene treated explants was essentially the same. Samples of 25 explants were placed either in air or stored in desiccators with 10 ppm ethylene. Samples were withdrawn and frozen every 3 hr for subsequent cellulase assays. As shown in Fig. 2, a 6-hr ethylene treatment caused an increase in cellulase activity compared to air-treated controls.

IAA, coumarin, and ^6N -benzyladenine block the ability of ethylene to stimulate abscission (6). The data in table III show that explants treated with these compounds produced less cellulase when exposed to 1 ppm ethylene. The 25 separation layers

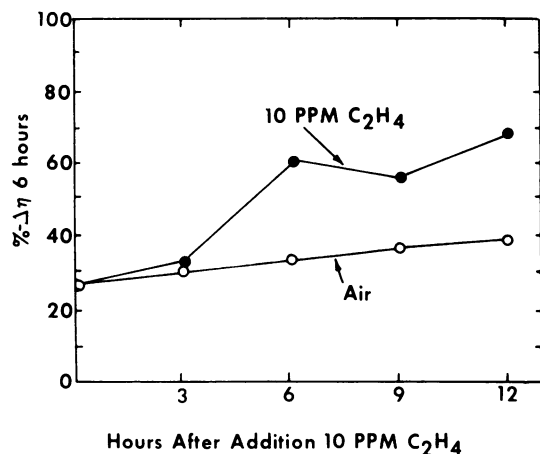


FIG. 2. Time course for induction of separation-layer cellulase in ethylene-treated and control explants. Explants aged for 20 hr were stored in desiccators containing air or 10 ppm ethylene. Samples were withdrawn as indicated and assayed for cellulase activity.

were homogenized in 4 ml of buffer in a Ten Broeck homogenizer, centrifuged at 10,000g for 10 min and then assayed for cellulase.

Since CO₂ competitively inhibits the ability of ethylene to accelerate abscission (3) and since cellulase is postulated as integral in the cell separation process, it should be possible to demonstrate that CO₂ can block the increase in cellulase activity associated with applications of ethylene. In these experiments 10 explants were inserted in agar in 40 ml flasks covered with cheesecloth. After 16 hr the bottles containing explants to be treated with ethylene and/or CO₂ were stoppered with rubber vaccine caps. Ethylene and CO₂ were injected into the gas phase surrounding the explants by means of a syringe inserted through the rubber vaccine cap. After 6 hr exposure to 1 ppm ethylene and/or 10% CO₂ the explants were removed from the bottles, their separation layers excised and frozen until subsequent ex-

Table III. Effect of 5×10^{-5} M IAA, 10^{-3} M Coumarin and 10^{-3} M ⁶N-Benzyladenine in the Presence of 1 ppm Ethylene on Cellulase Induction in Bean Explant Separation Layer

Groups of 25 explants were placed pulvinal end down in plain agar or agar containing abscission retardants for 24 hr. Except for controls which were left in air, the explants were placed in 1 ppm ethylene for 24 hr. The separation layers were then excised, frozen, and analyzed for cellulase activity. Initial activity represents separation layers from freshly excised explants.

Treatment	%-Δη 4 Hr
Initial	5
Control	77
C ₂ H ₄	91
C ₂ H ₄ + IAA	18
C ₂ H ₄ + Coumarin	6
C ₂ H ₄ + ⁶ N-Benzyladenine	32

Table IV. Effect of 1 ppm C₂H₄ and 10% CO₂ on Cellulase Formation in Bean Explant Separation Layer

Explants in groups of 10 were aged 16 hr in air, followed by 6 hr in the gas phase indicated in the table. Separation layers were excised, frozen, and analyzed for cellulase.

Treatment	%-Δη 16 Hr
0 Hr initial	14
Control	25
C ₂ H ₄	47
CO ₂	24
C ₂ H ₄ + CO ₂	21

traction with 3 ml of phosphate buffer in a Ten Broeck homogenizer. Table IV summarizes data which show that 10% CO₂ was able to overcome the ability of ethylene to increase cellulase activity.

Actinomycin D and cycloheximide have been used to demonstrate a requirement for RNA and protein synthesis in abscission (5). If cellulase is one of the proteins synthesized during abscission, it should be possible to reduce the amount of this enzyme by treating explants with actinomycin D and cycloheximide. Groups of 25 explants were injected with actinomycin D and cycloheximide as described in the methods section. The separation layers were extracted with 4 ml of buffer. The data in table V indicate that actinomycin D and cycloheximide inhibited the increase in cellulase activity by ethylene.

The preceding experiments demonstrate that cellulase activity was associated with cell separation of abscission zone explants. I have also found that cellulase activity (%-Δη after 3 hr = 39) occurred in the basal end (0.5 cm) of freshly abscising *Coleus* petioles while little or no activity (%-Δη after 3 hr less than 8) was present in the remaining petiole tissue or in intact leaves further up the stem.

Table V. Effect of Actinomycin D and Cycloheximide in the Presence of 1 ppm Ethylene on Cellulase Formation

After a 24-hr aeration period, groups of 25 explants were injected with actinomycin D and cycloheximide. The explants were then given an 8 hr 1 ppm ethylene treatment before excising and freezing separation layers.

Treatment	%-Δη 5 Hr
0 Hr initial	7
C ₂ H ₄	43
C ₂ H ₄ + Actinomycin D 1 μg	11
C ₂ H ₄ + Cycloheximide 0.25 μg	7

Discussion

The experiments described in table I confirm and extend to cotton and *Coleus* the findings of Horton and Osborne (14) that cellulase activity was associated with the separation layer of abscission zone explants undergoing abscission.

Separation-layer cellulase is a soluble enzyme (table II) with maximum activity at pH 7.0 and stable up to 40°. Fungal cellulases usually show optimum activity at pH 4.0 to 5.5 (15), tomato cellulase at 5.0 (10), snail cellulase at 5.6 (18), bacterial cellulase around 6.0 (12), and nematode cellulase from 5.5 to 8.0 (11). Abscission zone cellulase was not as stable as a crude preparation from *Irpex lacteus* and *Trichoderma viride* which retain 16 to 30% of their original activity after 30 min at 99° (13). Sison *et al.* (22) reported a 44% loss in activity of cellulase from *Poria vaillantii* after a 70° 10-min treatment, while Myers and Northcote (18) found that snail cellulase was rapidly inactivated at 30°.

Ethylene increased cellulase activity of aged separation layer cells after a 3-hr lag period (Fig. 2). These findings agree with the earlier observations that ethylene increased RNA synthesis after an hr lag period and protein synthesis after a 2-hr lag period (4). Data obtained with cycloheximide also suggested that proteins essential to abscission were synthesized after a lag period.

Results of experiments measuring the influence of aging retardants, CO₂, and the inhibitors, actinomycin D, and cycloheximide, on cellulase formation in the separation layer of bean explants agree with what was known on the action of these compounds on abscission. The aging retardants are thought to slow down or prevent the onset of the ethylene-sensitive stage of abscission (6). These compounds also prevented ethylene from inducing cellulase activity (table III). The gas CO₂, on the other hand, acts as a competitive inhibitor of ethylene in abscission (3) and, as shown in table IV, was able to overcome some of the effect of ethylene in increasing cellulase formation. Finally, actinomycin D and cycloheximide are known to inhibit abscission and presumably act by blocking RNA and protein synthesis required for the formation of degradative enzymes (5). As shown in table V, actinomycin D and cycloheximide blocked the induction of cellulase activity.

Control of cellulase activity by inhibitors of low molecular weight or activators could explain some of the results presented here. However, experiments designed to observe such control mechanisms have given negative results. For example, an inhibitor of cellulase activity might be present in unaged tissue and these materials could decrease with age. However, protein-free extracts of juvenile explants had no effect on the cellulase produced by aged explants. Conversely, dialysis or NH₄SO₄ precipitation of proteins from juvenile explants did not result in increases in cellulase activity. In other experiments the possibility that activators may play a role in cellulase activity was examined. In these experiments dialysis or NH₄SO₄ precipitation of aged explants did not reduce activity nor did protein-free extracts from

aged explants increase cellulase activity from unaged explants.

As discussed in greater detail in a recent review (2), abscission can be described by an aging-ethylene hypothesis. The essential features of this hypothesis as applied to explants is that excision of explants cuts off the supply of juvenility factors, such as auxin, normally supplied by the leaf. In the absence of these juvenility factors, the explants age and the separation layer becomes increasingly sensitive to ethylene. From work with RNA and protein metabolism, it was concluded that the mechanism of ethylene action during cell separation was to induce protein essential for the cell separation process. Both the work by Horton and Osborne (14) on cellulase induction during abscission and this report confirm this interpretation and indicate that the mechanism of ethylene action, like other hormones, is to regulate the production of enzymes that are essential for physiological processes.

Acknowledgments

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