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Cellulase and Abscission in the Red Kidney Bean (*Phaseolus vulgaris*)[1]

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ABSTRACT

Cellulase (β-1,4-glucan-glucanohydrolase EC 3.2.1.4) activity in the abscission zone of red kidney bean (*Phaseolus vulgaris*) was previously shown to exist in at least two different molecular forms. The form of the enzyme which has an isoelectric point of 4.5 is present in both abscising and nonabscising tissue and requires grinding for extraction. Another form of the enzyme which has an isoelectric point of 9.5 is present only in tissue in which the abscission process has been induced. Further, much of this form of cellulase can be removed from the tissue by vacuum infiltration with buffer. Time course studies indicate that while the increase in measureable cellulase activity in tissue which is actively undergoing abscission was due primarily to the appearance of cellulase 9.5, this form of the enzyme cannot be removed by vacuum infiltration until after the breakstrength of the abscission zone has decreased nearly to zero. The intracellular localization of these two forms of cellulase is discussed.

The role of cellulase in mediating the process of abscission has been difficult to determine even though several laboratories have been able to show a correlation between the activity of the enzyme and the event of abscission (1, 5, 9, 11, 13). This problem has been compounded by the recent report that cellulase exists in more than one molecular form (10, 11). Lewis and Varner (11) were able to demonstrate a correlation between cellulase activity and decrease in breakstrength of abscission zones of *Phaseolus vulgaris,* and further that the increase in activity as abscission proceeded was due to the *de novo* synthesis of a form of the enzyme which was more soluble in a high salt buffer than in the same buffer without added salt.

Lewis *et al.* (10) have reported that, in freshly harvested abscission zones, only an acidic form of cellulase could be extracted and that this cellulase was relatively unstable. After induction of abscission, the acidic form could still be detected as well as at least one other form of the enzyme which was a basic protein, as determined by isoelectric focusing. Further characterization of these two cellulases indicated differences in sensitivity to *p*-chloromercuribenzoate, mol wt, and stability at 50 C.

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This study reports our attempts to follow the activities of these two forms of cellulase during the process of abscission as measured by decreasing breakstrengths of abscission zones.

MATERIALS AND METHODS

Plant Material. Abscission zones were taken from the petiole segment just below the primary leaf blades of 10- to 12-day-old red kidney beans, *Phaseolus vulgaris.* For large experiments involving 1000 or more abscission zones, the plants were grown in the greenhouse at 27 ± 2 C in a peat-sponge rock mix and watered daily with 0.5 Hoagland's solution. Smaller amounts of tissue were taken from plants grown in a growth chamber at 25 C with 8 hr of light from both incandescent and fluorescent lamps at an intensity of 1000 ft-c, followed by 2 hr of incandescent light of 100 ft-c and 14 hr of dark. Chamber grown plants were grown in a vermiculite-gravel medium and watered daily with 0.5 Hoagland's solution.

Treatment of Explants. Explants were prepared by removing the primary leaf blades and the cotyledons, and the plant was cut off just above the soil leaving the main axis plus the petioles including the distal abscission zone of the primary leaves as a unit. Zero time (untreated abscission zones) were prepared by removing the distal abscission zone including the pulvinus plus 3 to 5 mm of petiole. Ethylene treatment was carried out by allowing the explants to sit in a beaker of water for 24 hr followed by exposure to 50 μl/l of ethylene gas in an airtight chamber.

Breakstrength. The breakstrength across the abscission zone was measured as the force necessary to separate pulvinus from petiole. Petioles were cut from the explant at the node. A strip of cheesecloth, 2.5 × 0.5 cm, was twisted around the petiole about 0.5 cm below the separation layer and clamped firmly with a hemostat. Secured petioles were suspended from a Chatillion-pull gauge. Tension force was applied manually by pulling the pulvinus until the tissue broke apart. This technique gave a repeatable indication of the abscission process between about 200 to 10 g. Forces greater than 200 g caused rupture of the tissue in places other than the separation layer and forces less than 10 g were difficult to measure.

Cellulase Extraction. Cellulase (β-1,4-glucan-glucanohydrolase EC 3.2.1.4) was routinely extracted by grinding the tissue in 20 mM sodium phosphate buffer fortified with 1 M NaCl, pH 6.1 (5–10 zones/ml) with a Waring Blendor or by first subjecting the tissue to vacuum infiltration and then grinding. Vacuum-infiltrated extracts were prepared by placing the abscission zones in high salt buffer in a 50-ml suction flask (5–10 abscission zones/ml). A vacuum was applied to the flask with an aspirator and then released two or three times until bubbles

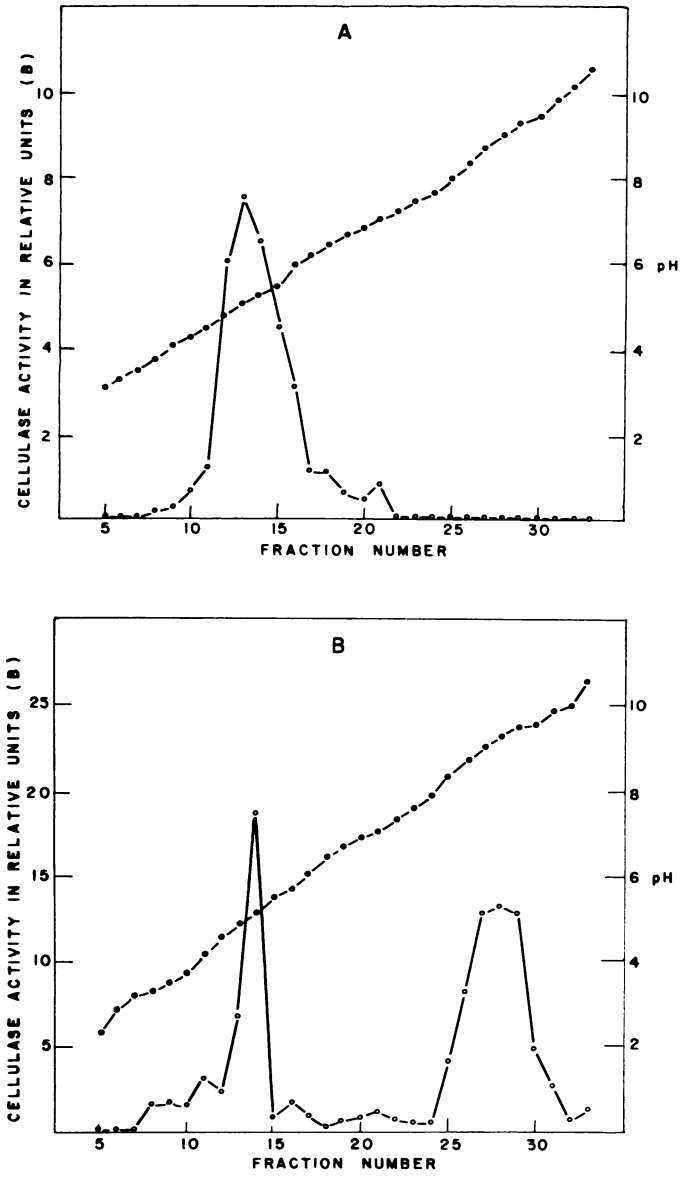

FIG. 1. Isoelectric focusing pattern of cellulase activity from freshly harvested and ethylene-treated abscission zones. Cellulase activity was extracted by grinding abscission zones in 20 mM phosphate buffer, pH 6.1, fortified with 1 M NaCl. Enzyme activity was focused in ampholine buffers (pH 3–10) at 600 v for 24 hr. Three-ml fractions were collected and adjusted to pH 6.1 before assay. A: cellulase activity extracted from zero time tissue. B: Cellulase activity extracted from abscission zones which had been treated with 50 μl/l of ethylene gas for 24 hr beginning 24 hr after preparation of explants.

were no longer emitted from the tissue (1–2 min). While cellulase could be directly assayed from the clear supernatant at this time, the flask was routinely placed on ice and gently shaken for 15 min after which time the tissue was removed, blotted dry, and then subjected to grinding as above. The extracts prepared by grinding were filtered through nylon cloth, and these extracts as well as those prepared by vacuum infiltration were centrifuged for 10 min at 10,000*g*. Extracts prepared by vacuum infiltration did not have a pellet either with zero time tissue or that which had been ethylene-treated. The extracts were then assayed and prepared for isoelectric focusing.

Table I. *Extraction of Cellulase from Red Kidney Bean Abscission Zones*

Cellulase was extracted by grinding in 20 mM sodium phosphate buffer, pH 6.1, with or without addition of 1 M NaCl. Estimation of per cent cellulase 9.5 is based on recovery of cellulase from isoelectric focusing.

Tissue	No. of Abscission Zones	Fresh Wt	Buffer Additions	Units		Estimated Cellulase 9.5
		g		*total*	*per zone*	%
Zero time	1228	33	No NaCl	583	0.48	0
			1 M NaCl	690	0.56	0
Ethylene-treated	996	27	No NaCl	1668	1.62	40–50
			1 M NaCl	6143	6.15	80–90

Enzyme Assay. Cellulase activity was assayed by measuring the reduction in viscosity of a solution of CM[2]-cellulose, type 7HF, (Hercules Powder Co., Wilmington, Del.). The assay mixture contained two parts of CMC-7HF, 1.2% (w/v) in 20 mM sodium phosphate buffer, pH 6.1, and one part of the enzyme solution. Drainage time through a calibrated portion of a 100-μl pipette was used as a measure of viscosity. Viscosity was usually measured after 1 to 2 hr reaction time. Enzyme reactions and viscosity measurements were done at room temperature of about 23 C. Viscosity data were converted to relative units of activity B/g·hr as described by Almin *et al.* (5) and extended to *Phaseolus* cellulase by Lewis and Varner (11).

Isoelectric Focusing. Isoelectric focusing was carried out as described by Haglund (8). The enzyme extracts were dialyzed against two changes of 1% glycine for 3 hr, combined with ampholine buffers (pH 3.0–10.0), and placed on an LKB Model 1801 isoelectric focusing apparatus. The proteins were focused for 36 hr at 600 v. Fractions collected from the column were then adjusted to pH 6.1 using 0.2 M Na_2HPO_4 or NaH_2PO_4. Each fraction was then assayed for cellulase activity. Percentage of recovery was calculated as the ratio of the total units recovered from the column to the total units put on the column after dialysis.

RESULTS

Isoelectric focusing of cellulase extracted from freshly harvested (zero time) abscission zones indicated that the enzyme had an isoelectric point of about pH 4.5 (Fig. 1A). After induction of abscission with ethylene, there was an increase in cellulase activity, and when this activity was subjected to isoelectric focusing, the majority of cellulase activity was recovered in two separate regions of the gradient (Fig. 1B). Lewis *et al.* (11) have referred to that form of cellulase which focuses between pH 4.2 and 4.6 as cellulase 4.5 and that which focuses between pH 9.2 and 9.6 as cellulase 9.5, and have partially characterized these two cellulases. Other minor peaks were consistently observed; however, they represent a small percentage of the total activity, and we have not made an attempt to study the properties of the other forms of cellulase at this time.

Attempts to determine whether cellulase 4.5 and cellulase 9.5 were differentially extractable indicated that a small but repeatable increase in cellulase activity (about 3-fold) could be extracted with the low salt buffer after abscission had been induced (Table I). A much greater increase (about 11-fold) in

[2] Abbreviations: CM: carboxymethyl; B: estimated arbitrary enzyme activity.

cellulase activity was observed in that cellulase which required high salt buffer for extraction. At zero time there was no detectable cellulase 9.5 by isoelectric focusing in either extract.

Table II. *Extraction of Cellulase from Kidney Bean Abscission Zones by Vacuum Infiltration*

Infiltration was followed by grinding in 20 mM phosphate buffer, pH 6.1, fortified with 1 M NaCl.

	Vacuum Infiltrated			Ground			Combined total		
	Units (U)	U/zone	U/g fresh wt	Units	U/zone	U/g fresh wt	Total U	No. of zones	U/zone
Zero time	91	0.06	2.8	3514	2.4	106.0	3605	1488	2.4
Untreated 48 hr	260	0.16	7.5	3234	2.0	93.7	3494	1634	2.1
Ethylene 48 hr	5624	3.2	131.0	7176	4.0	139.0	12818	1784	7.2

Since about half of the activity previously referred to as soluble cellulase (11) from ethylene-treated tissue was cellulase 9.5 and half cellulase 4.5, it does not appear that differential solubility results in a clear separation of these two molecular forms of cellulase.

Table II and Figure 2 present evidence which suggests that cellulase 4.5 may be primarily endocellular and that cellulase 9.5 is the form found outside of the membrane. Cellulase was extracted either before abscission was induced (zero time) or after an ethylene induction treatment by first placing the abscission zones in 20 mM sodium phosphate buffer fortified with 1 M NaCl and subjecting the tissue to vacuum infiltration. After shaking, the tissue was removed, and the clear supernatant was assayed for cellulase activity. The tissue was then blotted dry and ground in more of the high salt fortified buffer, centrifuged, and this supernatant was also assayed for cellulase activity. Cellulase extracted by these techniques resulted in recovery of more activity per abscission zone (2.4 units/zone) from zero time tissue than was recovered by the combination of grinding

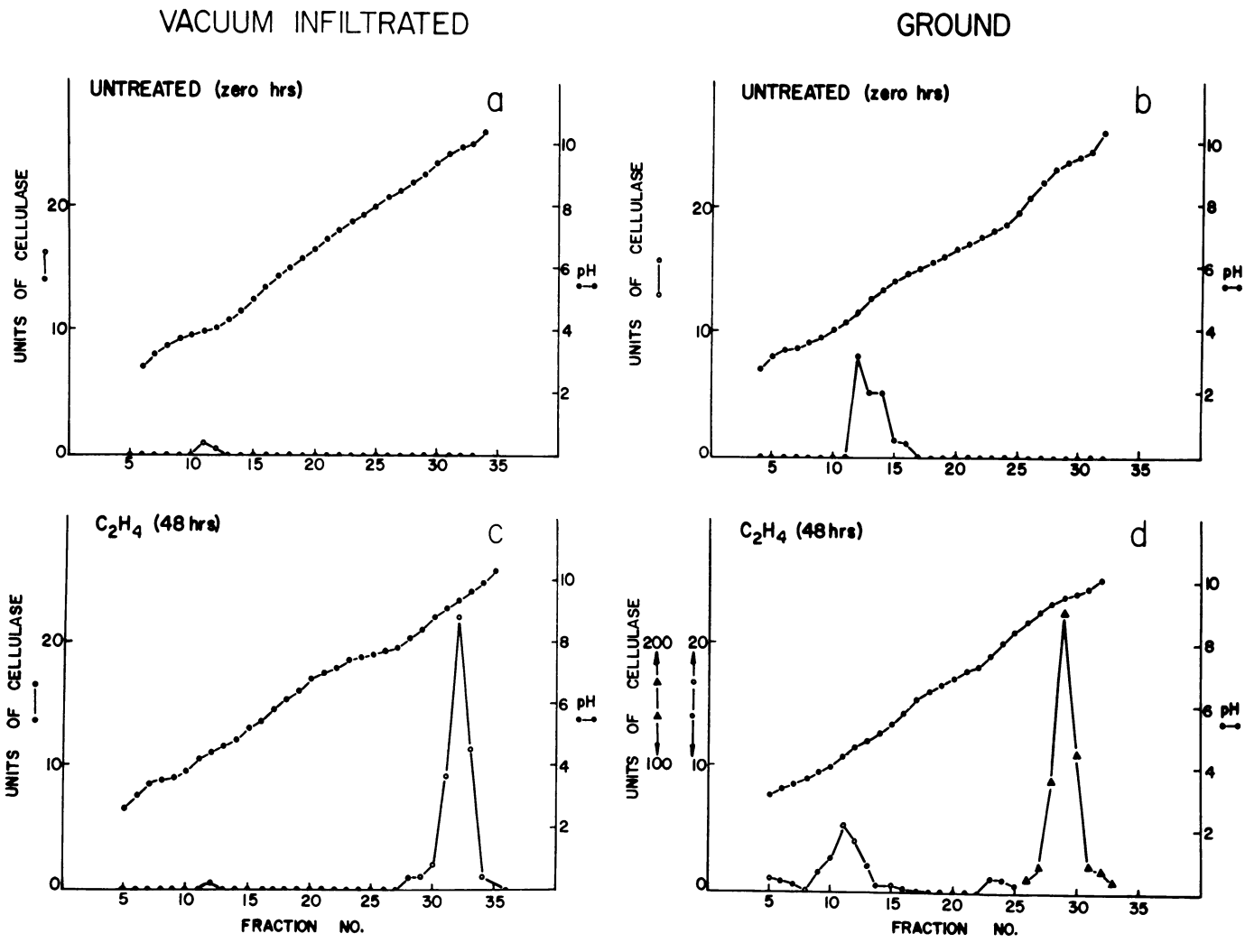

FIG. 2. Isoelectric focusing patterns of cellulases extracted by vacuum infiltration and by grinding nonabscising and ethylene-treated abscission zones. Cellulase was extracted from 200 abscission zones by first placing the segments in 20 mM phosphate buffer, pH 6.1, in a vacuum flask and decreasing the pressure with a faucet aspirator. Segments were then removed and ground in more of the same buffer. a: Isoelectric focusing pattern of cellulase activity extracted by vacuum infiltration technique from freshly harvested abscission zones. Ten per cent of the units put on the column were recovered. b: Isoelectric focusing pattern of cellulase activity extracted by grinding freshly harvested abscission zones after vacuum infiltration. Twenty per cent of the units put on the column were recovered. c: Isoelectric focusing pattern of cellulase extracted from ethylene treated abscission zones by vacuum infiltration. Thirty-five per cent of the units put on the column were recovered. d: Isoelectric focusing pattern of cellulase activity extracted by grinding ethylene treated abscission zones after vacuum infiltration. One hundred per cent of the units put on the column were recovered.

in low salt buffer followed by grinding in a high salt buffer as reported in Table I (1.04 units/zone). The amount of activity per abscission zone extracted from ethylene-treated tissue, however, was similar in both experiments.

Total cellulase activity (that extracted by vacuum infiltration plus that extracted by grinding) again showed about a 3-fold increase from ethylene-treated tissue over that extracted from zero time tissue (Table II). Only about 3% of the total activity found in zero time tissue could be extracted by the vacuum infiltration technique, whereas 44% of the total activity found in ethylene-treated tissue could be extracted by this method.

Extracts prepared by the vacuum infiltration grinding technique were placed on an isoelectric focusing column, and the results are indicated in Figure 2. Vacuum infiltration of the zero time abscission zones led to a release of a small amount of cellulase 4.5 (Fig. 2a). A greater amount of this form of the enzyme was obtained by then grinding this tissue (Fig. 2b). Again, cellulase 9.5 was not detected in the freshly harvested abscission zones. The increased cellulase activity which was extracted by vacuum infiltration of ethylene-treated abscission zones was primarily cellulase 9.5 (Fig. 2c). Grinding this tissue released even more cellulase 9.5 as well as the cellulase 4.5 activity (Fig. 2d).

Since the above results suggested not only that the increase in cellulase activity which correlates with the abscission process was due to the appearance of cellulase 9.5, but further that this form of the enzyme could be washed out of the abscission zones without disrupting the tissue by grinding, we then attempted to correlate the decrease in breakstrength of abscission zones with the appearance of cellulase 9.5.

Figure 3 shows the results of such an experiment. In this experiment, abscission was allowed to proceed slowly by standing the explants in a beaker of water on the laboratory bench. At intervals, samples were taken to test breakstrength of the abscission zones. Samples were also removed and cellulase extracted by both the vacuum infiltration and grinding techniques. The graph shows that a slight increase in cellulase activity did occur as the breakstrength decreased and continued to rise even after the breakstrength became essentially zero. However, cellulase 9.5 did not begin to appear until the abscission process was nearly completed (about 50 hr after excision). This activity also continued to increase after the abscission process was completed. The cellulase extracted in this experiment by vacuum infiltration was shown to be cellulase 9.5 both by isoelectric focusing and ion exchange chromatography using CM Sephadex (10).

Exposure to ethylene after the explants have been aged causes a much more rapid decrease in breakstrength (Fig. 4). The breakstrength decreased from about 200 g to zero in less than 10 hr. In tissue which has not been exposed to ethylene, a comparable decrease in breakstrength takes more than 40 hr (Fig. 3).

Figure 4 shows that even in tissue treated with ethylene, cellulase 9.5 does not appear in the fraction which can be washed out of the tissue without grinding until after the process of abscission has been completed.

DISCUSSION

The hypothesis that cell wall hydrolyzing enzymes, particularly cellulase, are involved in bringing about abscission has been suggested by several workers. Craker and Abeles (6) showed that cellulase increased prior to the morphological event of abscission in beans. Ratner *et al.* (13) found similar results in citrus. More recently, Lewis and Varner (11) were able to show only an inverse correlation between cellulase activity and breakstrength in bean abscission zones. The latter workers reported their data in relative units of enzyme activity rather than percentage of change in viscosity. The incubation time for the reaction was much shorter (1 hr *versus* 16 hr), and breakstrength was measured by pulling the zones apart.

One of the difficulties with this hypothesis has been that cellulase could be isolated from tissues which were not abscising. Since multiple forms of cellulase have been reported in fungi as well as bacteria (7), it is reasonable to speculate that multiple forms may also exist in higher plants.

Sheldrake (14) showed that cellulase activity extracted from different tissues of *Acer pseudoplatanus* have different pH optima and certain of these cellulases required the presence of detergent in the extraction medium to be solubilized. Lewis and Varner (11) presented evidence which suggested that there were two forms of cellulase in abscission zones of *Phaseolus vulgaris*,

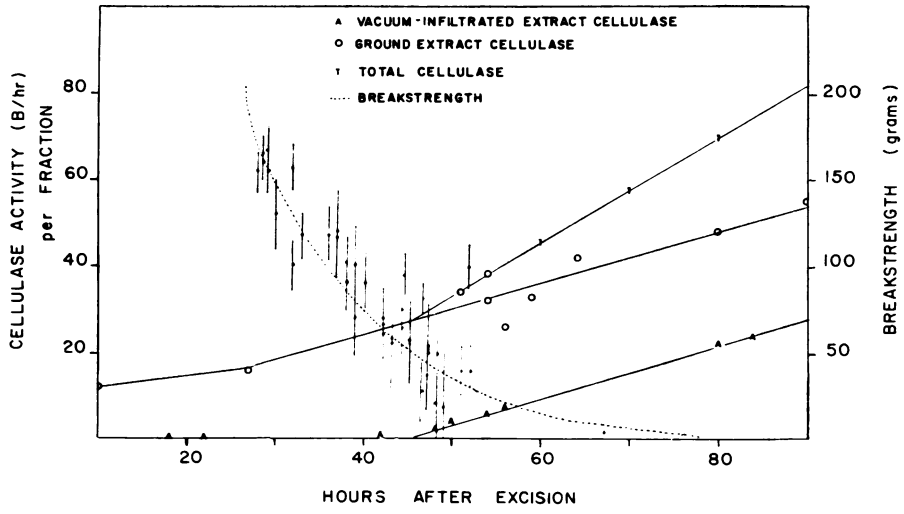

FIG. 3. Time course measurement of cellulase activity and breakstrength measurements from kidney bean abscission zones. Red kidney bean seedlings, 11 to 13 days old, were explanted and aged. Breakstrength (----) data points represent the average breakstrength of 20 abscission zones. Vertical lines represent 95% confidence intervals. Cellulase activity was extracted from 20 abscission zones by vacuum infiltration in 10 ml of cold 20 mM sodium phosphate buffer with 1 M NaCl, pH 6.1. The abscission zones were then removed and ground in 10 ml of the same buffer with a mortar and pestle with a pinch of purified sand. Both extracts were filtered through nylon cloth and centrifuged at 10,000*g* for 15 min before assaying cellulase activity.

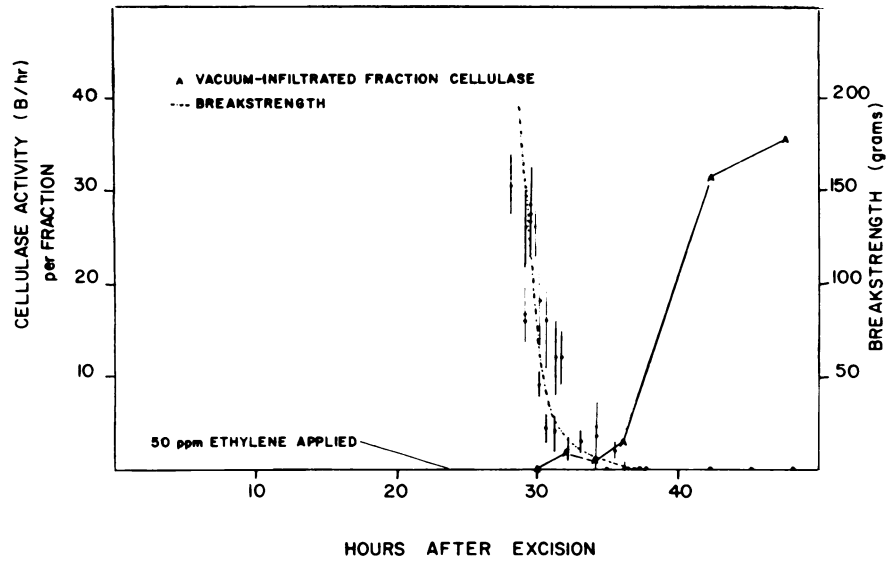

FIG. 4. Time course measurement of cellulase activity and breakstrength from ethylene-treated kidney bean abscission zones. Red kidney bean seedlings, 11 to 13 days old, were explanted and aged for 24 hr and then exposed to 50 μl/l of ethylene gas in a closed chamber. Breakstrength (----) data points represent the average breakstrength of 20 abscission zones. Vertical lines represent 95% confidence intervals. Cellulase activity was extracted from 20 abscission zones by vacuum infiltration in 10 ml of cold 20 mM sodium phosphate buffer with 1 M NaCl, pH 6.1. Cellulase extracted by this technique was shown to be almost exclusively (greater than 95%) cellulase 9.5 by isoelectric focusing.

one of which could be solubilized only with a high salt (1 M NaCl) fortified buffer. These authors further showed that the increase in cellulase activity following ethylene treatment was due to the appearance of the latter form and that it was synthesized *de novo* during this process.

Ahlgren *et al.* (3, 4) demonstrated that certain fungal cellulases had distinguishable isoelectric points. Using the technique of isoelectric focusing, we have been able to show that at least two forms of cellulase are present in abscission zones of red kidney beans which have been exposed to ethylene. These two forms have now been isolated and partially characterized (10). One form of the enzyme (cellulase 9.5) is not present in freshly harvested nonabscising tissue. The other form of the enzyme (cellulase 4.5) which can be extracted at zero time has the same isoelectric point as a form which can be extracted from ethylene treated tissue by grinding. However, it has not been established that the protein from zero time tissue is the same as that from ethylene treated tissue. In fact the cellulase activity isolated from zero time tissue has so far not been characterized since it appears to be less stable than the form which can be isolated after ethylene treatment.

The existence of intracellular, cell bound and extracellular forms of cellulase has been demonstrated for bacteria (15). Such localization of cellulase in higher plants has been more difficult to achieve. Recently, Abeles and Leather (2) were able to isolate cellulase activity from kidney bean abscission zones by first vacuum infiltrating the tissue with water followed by centrifugation which removed the liquid from the apparent free space of the tissue. Cellulase activity was demonstrated in this liquid and it was the activity of cellulase isolated by this procedure which was correlated with the decrease in breakstrength during abscission. This cellulase activity was called exocellular cellulase by these workers and its activity was compared to endocellular cellulase which they released by grinding the tissue. More recently, Rasmussen (12) has extracted cellulase from citrus by the same procedure.

A similar approach to isolating cellulase was attempted in our laboratory, incorporating modifications based on both the Lewis and Varner (11) suggestion that two separate cellulases could be distinguished by their different solubilities in low salt *versus* high salt buffers and the evidence of Abeles and Leather (2) regarding cellulase activity which could be washed out of the cell walls *versus* that which required grinding to be released. The data presented in Table II extend this idea by illustrating that in nonabscising tissue, very little cellulase could be washed out of the cell walls even if high salt buffer was used. Subsequent grinding of this same tissue released much more cellulase activity all of which appeared to be cellulase 4.5 (Fig. 2).

After abscission was stimulated by treatment with ethylene, a large amount of cellulase activity could be washed out by vacuum infiltration with the high salt buffer. It can further be seen that when this cellulase was placed on an isoelectric focusing column 99% of the activity recovered was cellulase 9.5. Subsequent grinding of this tissue released much more cellulase activity and both cellulase 4.5 and cellulase 9.5 were recovered from the isoelectric focusing column.

Since most of the cellulase 4.5 could be removed only by grinding of either zero time or ethylene-treated tissue, it is tempting to speculate that this molecular form may be intercellular (endocellular by the terminology of Abeles and Leather) and that the activity of this form does not change appreciably as abscission proceeds. However, since the recovery from the isoelectric focusing run was much less for the vacuum infiltrated extracts than for the extracts prepared by grinding, such a conclusion would disregard the possibility that losses in activity during isoelectric focusing could result in misinterpreting the data in Figure 2. For example, the losses in activity from freshly harvested tissue could be due to the disappearance of an unstable basic form of the enzyme. Such a result could not be detected by this technique.

Webster (16) has shown that as abscission occurs in this tissue there is both a breakdown of the cell walls in the abscission layer as well as extrusion of cell contents into the separation cavity. The cellulase activity which was removed by vacuum infiltration in abscising tissue could therefore be from the cytoplasm rather than from the extracellular region. In order to prevent this type of artifact, the abscission zones were thoroughly rinsed with distilled water prior to the enzyme extraction. Even after prior rinsing with distilled water there was a 50-fold increase in the amount of activity extracted by

vacuum infiltration from ethylene-treated *versus* zero time abscission zones.

It has also been suggested that one of the effects of ethylene may be to alter membrane permeability and that treatment with ethylene may result in the release of cellulase which was present within the plasmalemma prior to this treatment (2). Our results do not preclude this possibility; however, since cellulase 9.5 which appeared only after the onset of abscission is a smaller molecule than the cellulase 4.5 (10), it is possible that this form could more readily penetrate the plasmalemma and subsequently be removed from this tissue without grinding.

Since cellulase 9.5 could be removed by vacuum infiltration of abscission zone tissue and did not appear in any extract until abscission had been induced, it was tempting to speculate that this form of the enzyme may play a more direct role in mediating the abscission process than cellulase 4.5. Time course studies indicated, however, that the breakstrength had decreased to nearly zero before cellulase 9.5 appeared in the extracts prepared by vacuum infiltration (Figs. 3 and 4).

Our data suggest the hypothesis that the process of abscission as measured by decreasing breakstrength is not initiated by cellulase. It is possible that cellulase does play an important role in the final separation process since the sensitivity of our breakstrength measurements are not reliable below about 10 g. We would further suggest that the form of the enzyme we call cellulase 9.5 may be more directly involved in this final separation process than is cellulase 4.5.

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