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Changes in Two Forms of Membrane-Associated Cellulase during Ethylene-Induced Abscission[1]

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ABSTRACT

Only one form of membrane-associated cellulase was found previously in the lower petiolar pulvinus of *Phaseolus vulgaris* (cv Red Kidney). The cellulase has an isoelectric point (pI) of 4.5 (DE Koehler, LN Lewis 1979 Plant Physiol 63: 677–679). This enzyme was detected in abscission zones collected before the onset of abscission (control tissue), and was thought to represent a pre-secretory form of another cellulase, the abscission cellulase, which has a basic pI and is secreted during abscission. We now show that this acidic, membrane-associated cellulase is a glycoprotein, tightly bound to the membrane, with maximum activity at pH 5.1, and that it is not immunologically related to the abscission cellulase. Furthermore, when bean explants are induced to abscise with ethylene, the activity of the acidic cellulase declines rapidly to 50% of control levels in the first day. When abscission is fully developed, the membranes contain a basic form of cellulase with a pI of 8.0 to 9.0 and only trace levels of the acidic cellulase. The basic form is not a high mannose glycoprotein; it has maximum activity in a broad pH range (4.0–8.0) and is antigenically related to the abscission cellulase, which is induced during abscission and transported to the cell wall. Antibody raised against the abscission cellulase recognized two proteins in a crude membrane fraction from abscising tissue. One of those proteins comigrated with the abscission cellulase, and the other was 1 to 2 kilodaltons larger. Thus, during abscission, the acidic membrane-associated cellulase rapidly declines before the appearance of the abscission cellulase. We conclude that there is no conversion from the acidic cellulase to the basic cellulase and suggest that the acidic and basic cellulase isoenzymes are proteins derived from two different genes.

Previous work in our laboratory has shown that there are three distinguishable cellulase activities in the lower petiolar pulvinus of *Phaseolus vulgaris*. A soluble and a membrane-associated cellulase have been extracted from freshly harvested tissue (8, 11). The soluble form can be recovered from the tissue homogenate by using a low-ionic-strength buffer and is characterized by an acidic pI[3] of 4.5 (11, 15). The membrane-associated enzyme, which can be activated and released by solubilization of the membrane with Triton X-100, also has an acidic pI of 4.5 (9). The third form of cellulase, the abscission cellulase, is referred to as 9.5 cellulase, because it has a basic pI of 9.5 (11, 12, 15). This enzyme appears in the lower petiolar pulvinus only when this tissue has developed an abscission zone between the stem and the petiole. The abscission cellulase is one of several cell wall hydrolases synthesized and secreted into the abscission zone to dissolve the walls of the cells in the separation layer. Secreted enzymes are thought to be synthesized and processed in the lumen of the ER before secretion and it has been suggested that the latent, membrane-associated form of cellulase is a precursor of 9.5 cellulase. We have purified 9.5 cellulase and raised rabbit antibodies against it (10). This antibody was used to show that 9.5 cellulase increases concomitantly with a decline in the break-strength of the junction between the stem and the petiole (4).

In this study, we examined cellulases in membranes from abscission zones before and after abscission. We used 9.5 cellulase antiserum to determine if the acidic, membrane-associated cellulase from freshly harvested tissue is immunologically related to 9.5 cellulase. We determined by immunoblotting the occurrence of proteins related to 9.5 cellulase in the membrane fraction after abscission. Finally, we compared the enzymic and physical properties of the membrane-associated cellulases. We conclude that there is not a precursor-product relationship between these two proteins.

MATERIALS AND METHODS

Plant Material and Induction of Abscission. Lower petiolar pulvini of *Phaseolus vulgaris* L., cv Red Kidney bean, were collected from plants grown for 12 d in a greenhouse. Abscission was induced as described previously (4) by exposing 2-week-old, debladed bean explants to an ethylene air mixture of 50 μL/L passed at a rate of 2 L/min at 25°C. Length of exposure to ethylene was 48 h or as otherwise indicated in the text.

Preparation of Membranes. Abscission zones were homogenized at 4°C with a Polytron at medium speed for 30 s. The ratio of tissue to homogenization medium was 1:3. The homogenization medium contained 500 mM Sorbitol, 50 mM 3-(*N*-morpholino)propanesulfonic acid-KOH (pH 8), 10 mM EGTA, 5% PVP, 0.5% BSA, 4 mM salicylhydroxamic acid, 2 mM DTT, and 1 mM PMSF. The homogenate was filtered through a nylon mesh (50 μm), and was centrifuged sequentially at 3,000*g* for 5 min and 12,000*g* for 10 min in a Sorvall SS-34 rotor to remove cell debris and larger organelles, and at 89,000*g* for 45 min in a Sorvall T-865 fixed-angle rotor to obtain a crude membrane pellet and a membrane-free supernatant. The membrane pellet was suspended and washed three times in 10 mM phosphate buffer (pH 8.0) containing 0.5 M sorbitol and 2 mM DTT (buffer washed membranes) or was suspended and washed three times in 10 mM phosphate buffer (pH 8.0) containing 0.5 M sorbitol, 2 mM DTT, and 0.25 M NaCl (salt washed membranes). The material which was retained in the nylon mesh after cell disruption was reextracted with a high salt buffer (20 mM K_2HPO_4/KH_2PO_4 [pH 8.0], 0.5 M NaCl, 3 mM EDTA) and represents a cell-wall-enriched fraction.

Assays. Cellulase activity was determined by a viscosimetric

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[3] Abbreviations: pI, isoelectric point; PMSF, phenylmethyl-sulfonyl-fluoride; Con A, concanavalin A; SB_n, sulfobetaines with alkyl chain length of *n* carbons.

assay with 1.0% carboxymethyl cellulose (type 7H3SF, Hercules Powder Co.,) as substrate in 50 mM phosphate/citrate buffer at the indicated pH (12). To determine the total content of cellulase activity in abscission zones, we assayed the supernatant, the cell-wall-enriched fraction, as well as the membrane fraction.

Removal of 9.5 Cellulase from the Supernatant. Two mL of a membrane-free supernatant (200 units of 9.5 cellulase activity) isolated from abscised tissue were incubated with 100 μL of 9.5 cellulase antiserum for 2 h at room temperature with gentle agitation. Subsequently, 100 μL of *Staphylococcus aureus* suspension were added and allowed to stand for 10 min. The protein antibody complex was pelleted by centrifugation at 2000*g* for 10 min.

Immunoprecipitation. Membranes were suspended to a protein concentration of 4 to 5 mg/mL in a solution containing 1% (w/v) Triton X-100, 0.4% BSA, 1 mM PMSF, kept on ice for 30 min, and subsequently centrifuged at 117,000*g* for 20 min. The supernatant (500 μL) was incubated with 9.5 cellulase antiserum or normal serum (25 μL) for 24 h at 4°C, according to the method of Durbin *et al.* (4). The protein antibody complex was pelleted by addition of *S. aureus* suspension (20 μl) followed by centrifugation at 2,000*g* for 10 min. The supernatants were assayed for cellulase activity at pH 6.2 (4).

Sepharose-Con A Treatment. Washed membranes were pelleted by centrifugation at 89,000*g* for 30 min and suspended to a protein concentration of 4 to 5 mg/mL in a solution containing 1% Triton X-100 (w/v), 0.4% BSA (w/v), 1 mM PMSF. The mixture was kept on ice for 30 min and was then centrifuged at 117,000*g* for 20 min. Aliquots (400 μL) of the solubilized membrane preparation were incubated with the indicated aliquots of 4B-Sepharose-Con A beads (Sigma) in the presence or absence of 0.2 M methyl mannoside. After incubation at 0°C for 30 min, the beads were pelleted in a microcentrifuge for 10 s and the supernatant removed and assayed for cellulase activity. Incubation of the solubilized membrane preparation with 4B-Sepharose served as a control.

Preparative Isoelectric Focusing. Washed membranes were suspended in a solution containing 1% (w/v) Triton X-100, 1 mM PMSF to a final protein concentration of 1 to 2 mg/mL using a glass homogenizer. The mixture was kept on ice for about 30 min and then centrifuged at 117,000*g* for 20 min. Ten mL of the clear supernatant were mixed directly with the isoelectric focusing gel (30 mL), a procedure that has been used to load high protein concentrations (13). The gel was 2 mm thick and consisted of 1.6% (w/v) ampholytes pH 3.0 to pH 10.0, 6% acrylamide (30% T/2.6% C), 0.033% ammonium persulfate and 0.16% *N,N,N',N'*-tetramethylenediamine. Isoelectric focusing was performed in an LKB 2117 unit at 4°C. Focusing was initiated at 200 V and the voltage was increased by 200 V/h to 950 V at the end of 4 h. The gel was divided lengthwise into two strips 1 and 5 cm wide and each strip was sliced at 1 cm intervals along the pH gradient. The pH of the 1 × 1 cm slices was determined following addition of 1 mL of 50 mM KCl and incubation under vacuum for 2 h at RT. The 5 × 1 cm slices were each incubated in 5 mL of 5 mM Tris/Mes (pH 6.5), 2 mM DTT for 24 h at 4°C, and assayed for cellulase activity. Recovery of cellulase activity indicated that exposure of cellulase to the gel polymerization reaction did not extensively modify the enzyme (data not shown).

Gel Electrophoresis and Immunoblotting. Membrane proteins were analyzed by SDS-urea-PAGE using gradient gels 1.5 mm thick. The stacking gel was 5.5% acrylamide in 0.054 M Tris-0.027 M H_2SO_4 (pH 6.1). The resolving gel was 8 to 16% acrylamide in 0.42 M Tris-0.031 M HCL (pH 9.18) (2). Both the separation and the stacking gel contained 2 M urea. Membrane proteins were extracted with H_2O saturated phenol and precipitated with ammonium acetate in methanol as outlined by Hurkman and Tanaka (7). The pellet was solubilized in 8 M urea, 0.1 M DTT and 0.1 M Na_2CO_3 and mixed at a ratio of 2:1 with sample buffer containing, 5% SDS, 5 mM Tris/Cl (pH 8.0) and 10% mercaptoethanol. The upper electrode buffer was 0.04 M borate (pH 8.64) and the lower electrode buffer was 0.42 M Tris-0.031 M HCl (pH 9.18). After electrophoresis, gels either were stained with 0.125% Coomassie blue dissolved in 50% methanol, 10% acetic acid, or were electroblotted to nitrocellulose (0.2 μm Schleicher & Schuell) for 18 h at 1.5 amp and 15°C using 20% methanol, 96 mM glycine, 12.5 mM Tris, and 0.1% SDS as transfer buffer. Following transfer, the nitrocellulose sheet was incubated with cellulase antiserum (1:80 dilution) in 1% gelatin, 0.15 M NaCl, and 0.04 M Tris (pH 7.5). Immunodetection was done with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G following the instructions to the Bio-Rad Immunoblot kit supplied by the manufacturer.

Carbonate Treatment. Crude membrane fractions were suspended in 100 mM sodium carbonate (pH 11.5) and incubated at 0°C for 30 min. During the incubation the suspension was gently mixed using a glass homogenizer. The membrane suspension was centrifuged at 117,000*g* for 30 min, and both the pellets and the supernatants were recovered for further analysis (5). The pH of the supernatant was adjusted to pH 8.0 before cellulase activity determination.

Zwitterionic Detergents. Crude membrane fractions were incubated with 0.2% SB_n (Zwittergent detergent, from 3-08 to 3-14 Calbiochem, Behring Diagnostic) and assayed for cellulase activity at pH 5.1.

RESULTS

Membrane Cellulase and Total Cellulase Activity during Abscission. Membrane cellulase and total cellulase activities of the lower petiolar pulvinus were assayed as a function of time of exposure to ethylene (Fig. 1). Note that total cellulase activity is the sum of the membrane, soluble, and cell-wall-enriched fraction. Maximum membrane cellulase activity occurred before

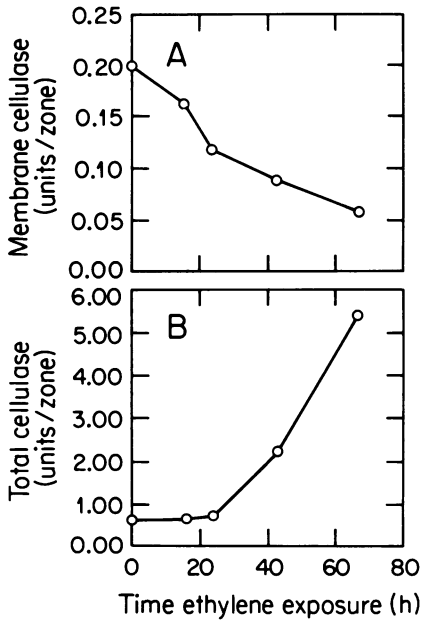

FIG. 1. Cellulase activity of abscission zones as a function of time in ethylene. Cellulase activity in the membrane fraction (A) and total extractable cellulase activity (B) during ethylene-induced abscission was assayed at pH 5.1. For each time point, 500 abscission zones were collected. Total extractable cellulase activity was the sum of activities from the soluble, membrane and cell-wall-enriched fractions.

ethylene treatment (Fig. 1A). During exposure to ethylene, membrane cellulase activity declined rapidly, decreasing over 50% in the first 30 h. Activity declined a further 25% in the next 48 h, during which time weakening of the abscission zone occurred. In contrast, total cellulase activity was low before ethylene treatment but began to increase rapidly after 24 h of exposure to ethylene attaining a 10-fold increase over the control after 72 h (Fig. 1B).

To test the possibility that soluble, inhibitory compounds caused the decrease in membrane cellulase activity (*cf.* 1), membranes from untreated tissue were incubated for 24 h at 4°C with the supernatant fraction from tissue treated with ethylene for 48 h, and then cellulase activity was assayed (Fig. 2). The supernatant fraction from ethylene treated tissue, however, contained high levels of cellulase activity (Fig. 2) contributed mainly by the basic cellulase with a pI of 9.5 (data not shown). Thus, before the membranes and the supernatant were mixed, the supernatant was depleted of 9.5 cellulase by immunoprecipitation with 9.5 cellulase antibody. The activity of the membrane cellulase did not change after incubation with increasing amounts of 9.5 cellulase-depleted supernatant. These results suggested that the decrease in membrane-associated cellulase activity after ethylene exposure was not caused by the appearance of a soluble inhibitor.

Isoelectric Focusing of Membrane Cellulases. The pI values of the membrane cellulases, before abscission develops and after 48 h of ethylene exposure, were determined using isoelectric focusing gels. Prior to the development of abscission, a membrane cellulase with an acidic pI of 4.5 to 5.0 was detected in the gel (Fig. 3A). After 48 h of ethylene exposure, activity of the acidic form was much lower, and a small peak with activity in the basic region was detected (Fig. 3B).

Interaction of Membrane Cellulases with Con A. Precipitation with Con A is commonly used to identify glycoproteins that have a high content of mannose groups (3). The binding of Con A to membrane cellulases was detected by the decrease in cellulase activity after incubation with Sepharose-Con A beads as described in "Materials and Methods." In control membranes, the activity of the acidic cellulase fell sharply upon addition of Sepharose-Con A beads (Table I). This activity was unaffected by Con A when 0.2 M methyl mannoside was included in the reaction mixture. No change in activity was observed when 4B-Sepharose alone was added. In contrast, addition of Sepharose-Con A to membranes from ethylene-treated tissue did not affect cellulase activity appreciably (Table I). The Con A sensitive cellulase activity present in control tissue remained associated with the membranes after three sequential washes with 0.25 M

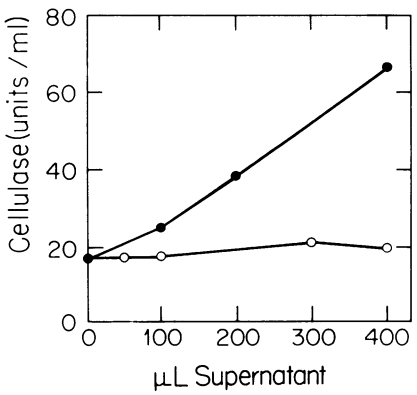

FIG. 2. Effect of supernatant from 48 h ethylene-treated plants on membrane cellulase activity from untreated tissue. Membranes were incubated for 24 h with increasing amounts of supernatant from ethylene-treated tissue, before (●——●) and after depletion (O——O) of 9.5 cellulase, and then assayed for cellulase activity.

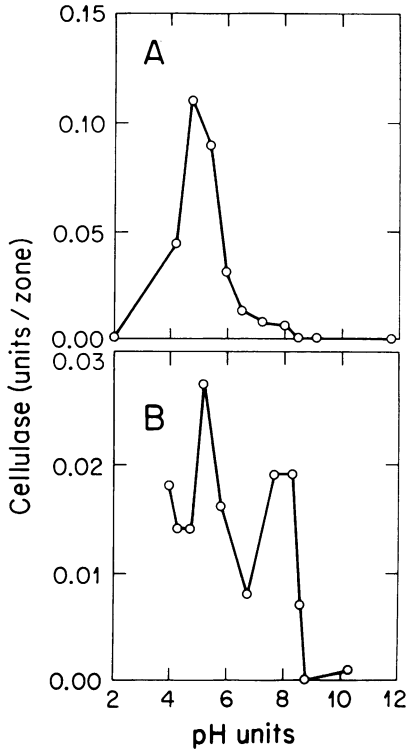

FIG. 3. Distribution of cellulase activity on preparative isoelectric focusing gel of solubilized membrane cellulases from control (A) and ethylene-treated (B) tissue.

NaCl (Table I). Furthermore, we determined whether Con A binds to the acidic cellulase eluted from the isoelectric focusing gel shown in Figure 3A. The activity of this acidic cellulase also fell sharply upon addition of Sepharose Con A (data not shown). This strongly suggests that the acidic cellulase is a membrane glycoprotein containing mannose oligosaccharide side chains.

Activity of the Acidic and the Basic Membrane Cellulases during Abscission. Once we established the differences between the acidic and basic membrane cellulases it was possible to follow the activity of each enzyme in the process of abscission. The acidic cellulase was distinguished by its Con A sensitivity and the basic cellulase by immunoprecipitation with 9.5 cellulase antiserum. The cellulase activity sensitive to Con A rapidly declined during the first day of ethylene exposure and continued to decrease to undetectable levels when abscission was fully developed (Fig. 4A). No cellulase activity was immunoprecipitated with 9.5 cellulase antiserum in membranes of untreated tissue (Fig. 4B). However, after 48 h of ethylene exposure, when the total membrane-associated cellulase activity was low (Fig. 1A), most of the cellulase activity was precipitated by the antiserum.

Immunoblotting With 9.5 Cellulase Antibody. Membrane and cell-wall proteins were analyzed by SDS-urea PAGE, before and after abscission developed. Immunoblots of the gels with antiserum to 9.5 cellulase showed that before ethylene exposure there was no 9.5-like protein associated with either the cell wall or the membrane fractions (Fig. 5A). After 48 h of ethylene exposure, there was one form of 9.5 cellulase associated with the cell wall fraction and two forms associated with the membranes, even after washing with 0.25 M NaCl. The cell wall form had an apparent M_r of 51 kD determined by PAGE, and was identical to the affinity purified abscission cellulase (9.5 cellulase) (Fig. 5B). The two forms associated with the membranes differ slightly in M_r. One form comigrated with the affinity purified abscission cellulase and the other form was 1 to 2 kD larger.

Table I. *Effect of Con A on the Membrane Cellulase from Control and Ethylene-Treated Tissue*

Membranes from control or ethylene-treated tissue were isolated and washed with either suspension buffer (10 mM phosphate [pH 8.0], 0.5 M sorbitol, and 2 mM DTT), or suspension buffer containing NaCl. The washed membranes were solubilized with Triton X-100 and incubated with Sepharose or Sepharose-Con A beads in the absence and presence of 0.2 M methyl mannoside. Cellulase activity was assayed after removing the beads by centrifugation.

Addition	Membrane Cellulase Activity			
	Control		Ethylene	
	Buffer wash	Salt wash	Buffer wash	Salt wash
	units/ml pH 5.1		*units/ml pH 6.2*	
Membrane alone	22.7[a]	23.2	2.0	9.2
4B-Sepharose, 200 μl	24.9	24.1	2.3	ND[b]
Sepharose-Con A, 200 μl	9.1	9.7	2.6	6.2
Sepharose-Con A, 200 μl + 0.2 M methyl mannoside	22.3	22.7	2.9	6.2

[a] Each point represents the average of two activity measurements. Results for control membranes correspond to the same membrane preparation, but results for membranes from ethylene treated tissue correspond to different membrane preparations. [b] ND, not determined.

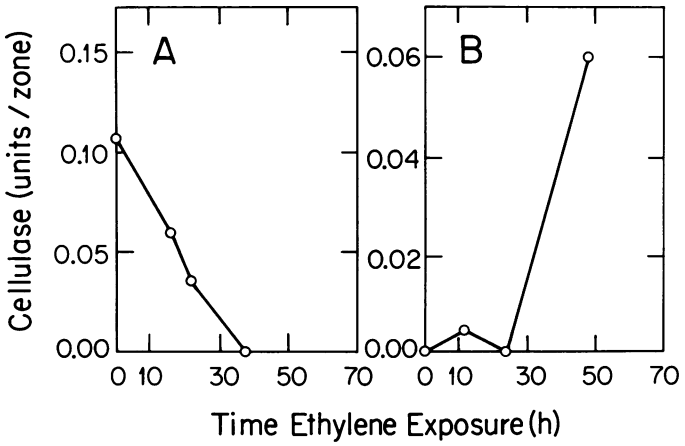

FIG. 4. Membrane cellulase activities as a function of time in ethylene. A, Con-A-precipitable cellulase. Membranes were solubilized using Triton X-100 and incubated with Sepharose-Con A beads in the presence and absence of 0.2 M methyl mannoside. The cellulase activity reported was the difference between the activities in the absence and presence of methyl mannoside. B, Immunoprecipitable cellulase. Membranes were solubilized as in A and were incubated with 9.5 cellulase antiserum. The cellulase activity reported is the difference between the activities with normal serum and 9.5 cellulase antiserum.

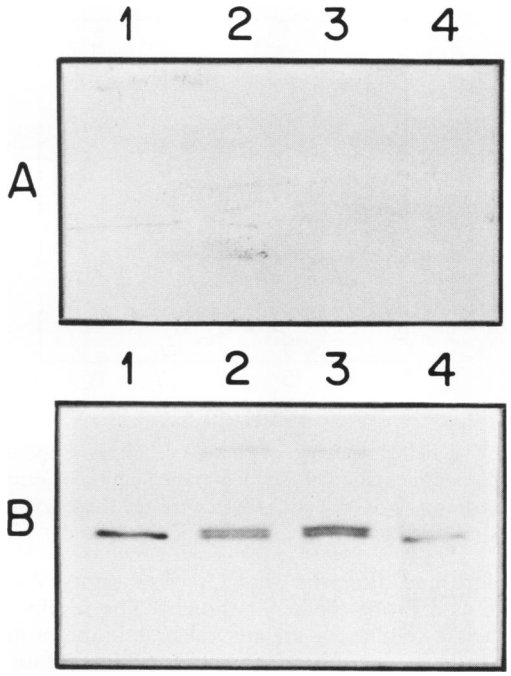

FIG. 5. Immunoblot of cell wall and membrane proteins from the lower petiolar pulvinus before (A) and after ethylene treatment (B) using 9.5 cellulase antiserum as a probe. Lane 1, cell-wall-enriched fraction; lane 2, buffer-washed membranes; lane 3, salt-washed; lane 4, membrane-free supernatant. The protein from the cell wall fraction and the membrane-free supernatant represent 50 abscission zones and the protein from the membrane fractions represents about 500 abscission zones.

pH-Activity Profile. The pH-activity profiles of cellulases from membranes isolated before and after abscission were compared. Figure 6 shows that the activity of membranes from untreated tissue was strongly pH-dependent, with an optimum between 4.5 and 5.1. In contrast, the pH dependence of the cellulase in membranes from ethylene-treated tissue was quite broad, with an optimum between pH 5.0 and 8.0. The comparison reveals a net decrease in activity across a broad range of pH after ethylene exposure. Subsequently the activity of the membrane cellulase from control tissue was assayed at pH 5.1. For the assay of membrane cellulase from ethylene-treated tissue, which has maximum activity over a broad pH range, we used a pH of 6.2 to conform with previous measurements of 9.5 cellulase activity (4, 11, 12).

The pH-dependent activity of the cellulases recovered from the supernatant fractions before and after abscission were also investigated. Figure 7 shows that the activity of the soluble cellulase occurring in control tissue (before ethylene exposure) had a pH optimum in a narrow range between pH 5.0 and 6.0, and the activity of the soluble cellulase detected in abscising tissue (after 48 h of ethylene exposure) had a pH optimum in a broad range between pH 5.0 and 8.0.

Na_2CO_3 Treatment. It has been reported that when organelles are diluted in ice-cold 100 mM Na_2CO_3 and then collected by centrifugation, closed membrane vesicles are converted to open membrane sheets and lumenal proteins, and peripheral membrane proteins are released in soluble forms (5). When the crude membranes from the abscission zone of control tissue were subjected to a carbonate wash, 75% of the acidic cellulase remained bound to the membranes. On the other hand, a carbonate wash of the membranes from ethylene-treated tissue extracted almost all cellulase activity. This result was confirmed by immunoblotting of the membrane proteins from ethylene-treated

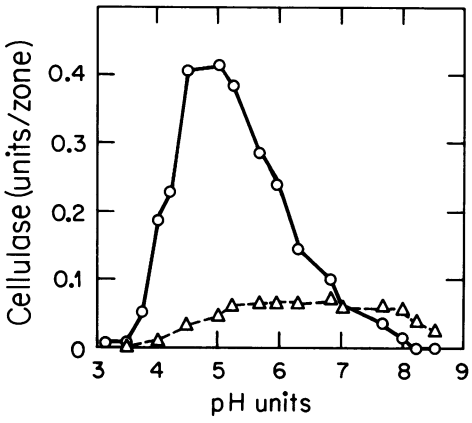

FIG. 6. The pH-dependence of membrane cellulases. Membranes were isolated from the lower petiolar pulvinus before (O——O) and after (Δ——Δ) exposure to ethylene. Cellulase activity was assayed as a function of pH.

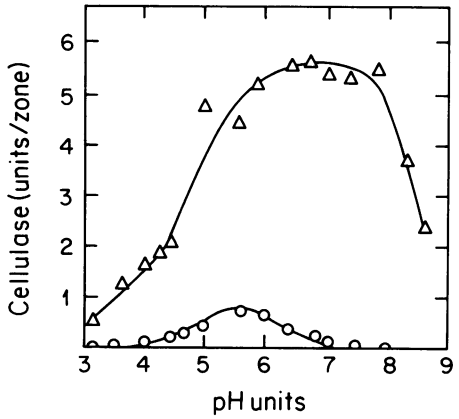

FIG. 7. The pH-dependence of soluble cellulases. Supernatants recovered after sedimentation of the membrane fraction by centrifugation from both control (O——O) and ethylene-treated tissue (Δ——Δ) were assayed for cellulase activity as a function of pH.

tissue before and after the Na_2CO_3 wash, using 9.5 cellulase antiserum as a probe (data not shown). The results suggested that the acidic cellulase is an integral membrane protein while the basic form is a peripheral protein or a lumenal microsomal protein.

Zwitterionic Surfactants. A homologous series of SB_n were examined for their ability to solubilize the acidic membrane cellulase. These compounds are a class of zwitterionic surfactants with different alkyl chain lengths. The SB_n of lower alkyl chain length ($n < 12$) can release only peripheral membrane proteins, while the SB_n of higher alkyl chain length ($n > 12$) can solubilize both peripheral and integral proteins. In addition, zwitterionic detergents are nondenaturating in their interaction with membrane proteins (6). When crude membranes were incubated with SB_n of $n = 8$, 10, 12, and 14, the corresponding cellulase activities were 4.4, 5.7, 7.2, and 20.9 units/mL, respectively.

DISCUSSION

Abscission is a process known to be induced by ethylene (17). During leaf abscission, cells from the separation layer produce hydrolases that lead to the dissolution of the cell wall and detachment of the leaf (16). However, little is known about the mechanism of secretion of the cell wall hydrolases. In general, proteins which are secreted from the cell are synthesized on polysomes bound to the ER and, after translation, the newly synthesized proteins are discharged into the lumen of the ER where processing begins (14).

Previous work in our laboratory demonstrated that one form of cellulase was associated with the plasma membrane of abscission cells (9). The enzyme was detected in abscission zones before the onset of abscission and had an acidic pI (9). Koehler *et al.* (8, 9) initially speculated that this enzyme could represent the abscission cellulase in a latent form and that secretion would lead to the conversion of the acidic cellulase to the abscission cellulase, which has a basic pI. In this study, we found that the acidic membrane cellulase is a distinct cellulase and does not share a precursor-product relationship with the abscission cellulase. The acidic membrane cellulase is a glycoprotein, as determined by the ability to bind to Con A. We discarded the possibility that the acidic cellulase could be bound to Con A through a complex with another glycoprotein, because the enzyme retained the Con A binding sensitivity after the membrane proteins were resolved by isoelectric focusing. This enzyme has a strong pH-dependent activity with an optimum at pH 5.1, and it is not immunologically related to the abscission cellulase (9.5 cellulase). The antiserum to the 9.5 cellulase did not precipitate the acidic membrane cellulase or recognize any protein after immunoblotting of the membrane proteins from control tissue. The acidic membrane cellulase from untreated tissue appears to be an integral membrane protein, because it cannot be extracted by 0.25 M NaCl, by a Na_2CO_3 wash, or by zwitterionic detergents of short alkyl chain length. The enzyme represents 40% of the total cellulase activity in the pulvinus during normal growth conditions. However, after exposure to ethylene, the acidic membrane cellulase rapidly declines to less than 50% in the first 24 h. It also appears that the decrease in the acidic membrane cellulase activity occurs prior to ethylene-induced synthesis of the abscission cellulase (9.5 cellulase).

Both the soluble and the membrane-bound, acidic cellulases extracted from untreated tissue are glycoproteins as determined by the ability to bind Sepharose-Con A. Both enzymes have similar activity-pH dependence and they differ from the activity-pH dependence of the basic cellulase that develops during the process of abscission. This property can be used as a simple tool to distinguish the acidic from the basic cellulase isoenzymes in a total tissue extract. The similarities between both the soluble and membrane-bound acidic cellulases suggest that they could be the same enzyme. It is worth noting that originally the soluble acidic cellulase was detected in low concentrations in stems, petioles, shoots and abscission zones from freshly harvested plants. These extracts were centrifuged for only 10 min at 10,000*g*, and therefore contained membranes (11).

When abscission has developed, membranes from abscission zones contain a cellulase that focuses between pH 8.0 and 9.0 and only trace levels of the acidic form. The basic form is not a high mannose glycoprotein; it shows a broad pH-dependent activity between pH 4.0 and 8.0; and it is immunoprecipitated by 9.5 cellulase antiserum. The results suggest that, after exposure to ethylene, the membranes acquire a new form of cellulase which is immunologically related to the 9.5 cellulase that is being secreted during abscission (12). Upon analysis of the membrane proteins of abscising tissue on SDS-urea-PAGE, followed by immunoblotting with the antisera raised against 9.5 cellulase, we found two proteins that were immunologically related to 9.5 cellulase. The two forms differed slightly in M_r. The low M_r form was identical to the 51-kD, 9.5 cellulase isolated from the cell wall fraction of abscising tissue and the larger form was 1 to 2 kD larger. The smaller form probably represents mature 9.5 cellulase associated with microsomal membranes due to nonspecific adsorption of a high pI protein to the membranes. In fact, a significant proportion of 9.5 cellulase was recovered in the

soluble fraction during preparation of membranes from abscising tissue. Furthermore, the only treatment that released all forms of 9.5 cellulase from the membranes was washing with Na_2CO_3 at pH 11. The larger form could represent a presecretory form of 9.5 cellulase, which is associated with the endomembrane before secretion. Using the same antiserum, Durbin *et al.* (4) quantitated the total amount of the abscission cellulase during the process of abscission. They demonstrated that there was a concomitant increase in cellulase activity and immunoprecipitable 9.5 cellulase protein that was inversely correlated with a decline in break-strength and that the enzyme was undetectable before abscission developed. Furthermore, when abscission developed while the explants were in ^2H_2O, a 1.25% increase in the buoyant density of cellulase in a cesium chloride gradient was observed (12). These data indicated that the increase in cellulase activity during abscission was a result of *de novo* synthesis of the abscission cellulase (12). Their findings, with the results presented here, indicate that 9.5 cellulase is synthesized *de novo* and that there is no conversion from the acidic cellulase to the basic cellulase. All these data support the concept that the 4.5 and the 9.5 cellulase are two different proteins derived from two different genes.

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