Expansin Mode of Action on Cell Walls[1]

Analysis of Wall Hydrolysis, Stress Relaxation, and Binding

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The biochemical mechanisms underlying cell wall expansion in plants have long been a matter of conjecture. Previous work in our laboratory identified two proteins (named "expansins") that catalyze the acid-induced extension of isolated cucumber cell walls. Here we examine the mechanism of expansin action with three approaches. First, we report that expansins did not alter the molecular mass distribution or the viscosity of solutions of matrix polysaccharides. We conclude that expansins do not hydrolyze the major pectins or hemicelluloses of the cucumber wall. Second, we investigated the effects of expansins on stress relaxation of isolated walls. These studies show that expansins account for the pH-sensitive and heat-labile components of wall stress relaxation. In addition, these experiments show that expansins do not cause a progressive weakening of the walls, as might be expected from the action of a hydrolase. Third, we studied the binding of expansins to the cell wall and its components. The binding characteristics are consistent with this being the site of expansin action. We found that expansins bind weakly to crystalline cellulose but that this binding is greatly increased upon coating the cellulose with various hemicelluloses. Xyloglucan, either solubilized or as a coating on cellulose microfibrils, was not very effective as a binding substrate. Expansins were present in growing cell walls in low quantities (approximately 1 part in 5000 on a dry weight basis), suggesting that they function catalytically. We conclude that expansins bind at the interface between cellulose microfibrils and matrix polysaccharides in the wall and induce extension by reversibly disrupting noncovalent bonds within this polymeric network. Our results suggest that a minor structural component of the matrix, other than pectin and xyloglucan, plays an important role in expansin binding to the wall and, presumably, in expansin action.

Because the plant cell wall behaves as a tough polymeric network to restrain cell turgor, wall yielding is generally recognized as a key control point for plant cell enlargement. Numerous biochemical and biophysical studies have indicated that wall yielding is the major means by which hormones and other agents exert their action on cell size (reviewed by Taiz, 1984; Cleland, 1986; Cosgrove, 1987, 1993). Despite considerable interest, the biochemical mechanism for cell wall yielding has long remained enigmatic. Matrix polysaccharides are believed to play an important

role in cell wall expansion as "tethers" to bind cellulose microfibrils together (Fry, 1989) or as "fillers" to prevent microfibril aggregation (McCann et al., 1990; Jarvis, 1992; Talbott and Ray, 1992). The problem of cell expansion then devolves to the mechanism by which the load-bearing matrix polymers shear during wall yielding and extension. According to most contemporary theories, hydrolases of xyloglucans (in dicots) or β-(1–3,1–4) mixed linkage glucans (in grasses) act as agents for wall loosening and turgor-driven expansion (Labrador and Nicolas, 1985; Fry, 1989; Hayashi, 1991; Inouhe and Nevino, 1991; Sakurai, 1991; Carpita and Gibeaut, 1993). More recently, the notion of transglycosylases as wall-loosening enzymes has also attracted attention (de Silva et al., 1992; Farkas et al., 1992; Fry et al., 1992; Nishitani and Tominaga, 1992). However, none of these enzymes has yet been shown to cause extension of isolated walls (McQueen-Mason et al., 1993), leaving room for doubt about their role in wall extension and cell enlargement.

Recently, we isolated, from growing cucumber (*Cucumis sativus* L.) hypocotyl walls, two proteins that induce the extension of isolated walls (McQueen-Mason et al., 1992). Here we refer to these proteins as "expansin 29" and "expansin 30" (abbreviated to Ex29 and Ex30, with respect to their relative molecular masses). Expansins can restore "acid growth" activity to heat-inactivated cell walls, but the biochemical mechanism of their action is uncertain. Expansins appeared to lack exoglycanase activity and xyloglucan endotransglycosylase activity (McQueen-Mason et al., 1992, 1993), but other possible mechanisms of action have not been explored.

In the present work we studied the action of expansins using three different approaches. First, we examined the possibility that cucumber expansins might function as endo-type hydrolases of matrix polysaccharides. Second, we analyzed the pattern of wall stress relaxation induced by these proteins. Finally, we studied the binding of expansins to the wall and its components as a way of identifying the wall components on which expansins may act to cause extension. Our results indicate that expansins act in a reversible (nonhydrolytic) manner on a matrix polymer that is tightly bound to the surface of cellulose microfibrils.

Abbreviations: CDTA, 1,2-cyclohexanedinitrotetraacetic acid; Ex29, expansin 29; Ex30, expansin 30; PAW, phenol:acetic acid: water (2:1:1, v/v).

The major components of the matrix, pectins and xyloglu-cans, do not appear to the site of expansin binding.

MATERIALS AND METHODS

Plant Materials

Cucumber (*Cucumis sativus* L. cv Burpee Pickler) seed-lings were grown in darkness for 4 d at 27°C as described by McQueen-Mason et al. (1992). For extension measure-ments, apical 3-cm hypocotyl sections were excised and stored frozen at −20°C for no more than 5 d. For wall extractions and fractionation, 6-cm hypocotyls were har-vested in bulk (typically in lots of 400 g fresh weight) and prepared as described below.

Extension Assay

Extension assays were performed as described by McQueen-Mason et al. (1992) using a constant load exten-someter. In brief, frozen/thawed hypocotyl sections were abraded briefly with carborundum, inactivated by boiling in water for 15 s, and secured between two clamps about 5 mm apart under a constant tension of 20 g. For most experiments wall specimens were surrounded with a plas-tic cuvette, allowing them to be bathed in appropriate solutions. Walls were initially bathed in 50 mM sodium acetate, pH 4.5. After about 30 min the solution was re-placed with a solution containing protein fractions of interest. Extension was detected using an electronic dis-placement transducer and recorded by a microcomputer (Cosgrove, 1989). Expansin activity was assayed as the increase in extension rate after adding protein.

For acid-induced extension assays, hypocotyls were pre-pared as above but not boiled. Extension was measured in the extensometer, initially with the walls bathed in 50 mM Hepes, pH 6.8; after about 30 min the bathing solution was exchanged for 50 mM sodium acetate, pH 4.5. Acid-induced extension was measured as the increase in extension after the switch to acid pH.

Protein Preparation

Proteins were prepared and separated as described by McQueen-Mason et al. (1992). Briefly, apical 6-cm hypo-cotyl sections were homogenized in 25 mM Hepes, pH 7.0; wall fragments were retained by filtration through Mira-cloth (Calbiochem, La Jolla, CA) and thoroughly washed in the same buffer. Proteins were solubilized from washed wall fragments in 20 mM Hepes, pH 6.8, 1 M NaCl and precipitated by addition of 390 g/L of $(NH_4)_2SO_4$. The precipitate was resuspended and separated by sequential HPLC, first using a C3 hydrophobic-interactions column and second by cation-exchange chromatography on a sul-fopropyl column (McQueen-Mason et al., 1992). The cation-exchange separation yielded two fractions with extension-inducing activity, referred to as S1 and S2 with respect to their order of elution. Active fractions from the C3 separa-tion (C3) were used directly as eluted from the column (in 50 mM sodium acetate, pH 4.5). Fractions S1 and S2 (which contain Ex29 and Ex30, respectively) from the cation-

exchange separation were used as they eluted from the column (15 mM Mes, pH 6.5, with some NaCl present) with the pH adjusted to 4.5 using 1 M acetic acid. Protein con-centrations were estimated using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and a calibration curve constructed with standard concentrations of BSA (Pierce).

Wall Fractionation and Preparation of Polysaccharides

Etiolated cucumber hypocotyl cell walls were fraction-ated in a sequential manner, with general procedures adapted from Fry (1988). Washed cucumber hypocotyl wall fragments (about 50 g wet weight) that had been extracted three times with 1 M NaCl (as described in protein frac-tionation method above) were extracted by stirring over-night at room temperature in 500 mL of PAW to remove extractable proteins. PAW wall fragments were strained through Miracloth and rinsed with ethanol until the phenol was removed. Wall residues were then dried and resus-pended in 500 mL of 50 mM CDTA, adjusted to pH 7.0 with NaOH, and stirred overnight. Wall fragments were re-tained by filtration through Miracloth. The CDTA extract was dialyzed over a period of 24 h against five changes of distilled water. The dialyzed extract was then frozen, ly-ophilized, and designated as the Pectin fraction (this frac-tion may also contain other polymers in minor abundance; Fry, 1988). The CDTA-extracted wall residues were washed in deionized water and then in ethanol before being dried under vacuum. Wall residues were resuspended in 400 mL of 4 M NaOH, 1% $NaBH_4$ and stirred overnight at room temperature. Wall residues were retained on a fluorocar-bon mesh (Spectra/Mesh Fluorocarbon, 105-μm mesh opening; Spectrum, Houston, TX), thoroughly washed in deionized water and then ethanol before drying in vacuo, and are described as 4M Walls. The extract solution was neutralized with glacial acetic acid and then dialyzed and lyophilized as described above. The dried extract was des-ignated as the Hemi-4 fraction. Wall residues were then stirred overnight in 6 M NaOH, 4% boric acid. Wall resi-dues (6M Walls) were retained on the fluorocarbon mem-brane and washed and dried as described above. The ex-tract solution was neutralized, dialyzed, and lyophilized as described above and is referred to as the Hemi-6 fraction.

4M Walls were then subjected to a series of different treatments. 4M Walls (250 mg) were incubated in 20 mL of 2 M TFA at 120°C for 1 h; wall residues were subsequently retained on Miracloth, washed with deionized water and then ethanol prior to being dried, and designated as TFA walls. Separate 4M Walls were incubated in 2 mg/mL pronase (Calbiochem, San Diego, CA), 50 mM sodium ac-etate, pH 5.0, 1 mM DTT, for 10 h at 25°C. Wall residues were retained on Miracloth and washed and dried in the usual manner. Another sample of 4M Walls was incubated for 10 h in 2 mg/mL cellulase (from *Trichoderma viride*; Boehringer Mannheim, Indianapolis, IN), and the wall res-idues were subsequently washed and dried. 4M Walls (1 g) were treated in 0.5% $NaClO_2$, pH 3.4 (with acetic acid), at 60°C for 15 min before being washed and dried. Finally, 4M Walls were given three consecutive 24-h incubations in 6 M

NaOH, 4% boric acid before being washed and dried; these are referred to as 3(6M) Walls.

Nasturtium xyloglucans were prepared from seeds of *Tropaeolum majus* L. as described by McDougall and Fry (1989). This method was also modified for the production of cucumber xyloglucans. Briefly, 1 g of Hemi-4 hemicelluloses (described above) was dissolved in 80 mL of water plus 10 mL of Fehling solution B and heated at 100°C for 30 min; after the solution cooled, 1 mL of Fehling solution A was added to precipitate the xyloglucans. Subsequent recovery of the precipitate and removal of copper was carried out as described by McDougall and Fry (1989).

Xyloglucan Molecular Mass Distributions

The Hemi-4 fraction of cucumber hemicellulose (above) was dissolved at a concentration of 5 mg/mL in 1 mL of 15 mM Mes, pH 6.5, with no protein addition (control) or 5 μg of S1 protein or 5 μg of S2 protein. The solutions were adjusted to pH 4.5 with 1 M acetic acid and incubated for 4 h at 30°C. After incubation, 0.2 mL of 0.5 M NaOH was added to each incubation mixture, and they were loaded separately onto a 75-mL column of Sepharose CL-4B (Pharmacia LKB) and eluted with 0.1 M NaOH at a flow rate of 1 mL/min and collected in 2-mL fractions. Fractions were acidified with 220 μL of 1 M acetic acid, and 0.5-mL aliquots were assayed for xyloglucan with the iodine method of Kooiman (1960).

Viscometric Assays

About 50 mg of cucumber Hemi-4 or pectins (as described above) were suspended in 1 mL of 50 mM sodium acetate, pH 4.5, and vortexed for 5 min, and the insoluble matter was removed by centrifugation at 12,000g in a microcentrifuge. Pectin or hemicellulose solutions were mixed with 0.1 mL of the relevant protein solutions (pH adjusted to 4.5 with 1 M acetic acid where necessary) or with 0.1 mL of buffer to give a final volume of 0.7 mL. The viscosity of the solutions was measured periodically in a custom-made rolling ball viscometer (Block and Spudich, 1987) with time measured with a hand-held stop watch. The viscometer was calibrated with Suc solutions of known viscosity.

Stress-Relaxation Measurements

Wall specimens were held between two clamps (5 mm between jaws) in a custom tensile tester as described by Cosgrove (1989). The tissue sample was extended at a rate of 170 mm/min until a stress of 20 g was attained and then held at constant strain. Stress was recorded over 5 min by a microcomputer with a minimum sampling rate of 2 ms, gradually increasing to 2 s. The relaxation spectrum was calculated as the derivative of the stress with respect to log (time).

Coating of 3(6M) Walls or Cellulose with Soluble Polysaccharides

Soluble cucumber wall matrix polysaccharides (prepared as described above), xylans (No. X-0376, from oat spelts,

Sigma), β-glucan (No. G-6513, from barley, Sigma), cucumber xyloglucans, and nasturtium xyloglucans (preparation described above) were suspended by stirring 200 mg in 10 mL of H₂O for 3 h at 25°C. Solutions were centrifuged at 10,000g for 10 min. The insoluble pellets were discarded and the supernatants dried under vacuum. Fifty milligrams of the dried soluble polysaccharides were dissolved by stirring in 5 mL of 25 mM sodium acetate, pH 4.5, for 30 min at 25°C, and 100 mg of either 3(6M) Walls or cellulose (No. C-6288, fibrous, medium; Sigma) were added and stirred for a further 60 min. The suspensions were dried overnight in an oven at 80°C, resuspended by stirring in 10 mL of H₂O for 1 h, and centrifuged at 10,000g for 10 min. The supernatant was discarded, and the pellet was resuspended in an additional 10 mL of water and again centrifuged to remove unbound polysaccharides. The supernatant was discarded and the coated cellulose or 3(6M) Walls (pellets) were dried under vacuum.

Extraction of Expansins and Acid-Induced Extension

Apical 1-cm sections of cucumber hypocotyls, which had been frozen, thawed, and abraded, were pressed between two glass plates to remove excess fluids. For each treatment eight hypocotyl sections were extracted in 2 mL of solution. Extraction solutions were all made up in 50 mM sodium acetate, pH 4.5, and contained one of the following; 1 M NaCl, 1 M urea, 1 M guanidine HCl, 0.2% Triton X-100, or 0.2% SDS; control extraction was in buffer only. After 1 h of extraction the solution was removed and transferred to a Centricon-30 microconcentrator (Amicon, Beverly, MA). After concentration, the solution was exchanged three times for deionized water on these filters to remove salts and detergents. Proteins were recovered from the filters and brought to a total volume of 0.5 mL in 20 mM Tris, pH 7.0. Serial dilutions of 1:10 and 1:100 were made, and three 0.1-mL aliquots of each dilution were used in ELISAs. Extracted stem sections were given three 10-min rinses in 50 mM sodium acetate, pH 4.5, before being tested for acid-induced extension in extensometer assays. Wall specimens were subsequently rinsed in deionized water, dried, and weighed.

Binding Assays

C3 proteins (20 μg/mL) were mixed with various cell wall fractions (preparation described above) at final concentrations of 10 mg/mL. These mixtures were then tested in the extension assay.

Quantitative Binding Assays

Purified expansin fractions S1 and S2 were prepared as described above and then concentrated on Centricon-30 microconcentrators. Proteins were quantified by UV light A_{280} and by Coomassie Protein Assay Reagent, using BSA for calibration. Aliquots of the S1 and S2 fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue R250. This was used to determine the proportion of the Coomassie staining attributable to the expansin band (in these experiments 50–75%) and hence to estimate the

quantity of expansins in the fractions. A fine suspension of 4M Walls in H_2O (1 mg/mL) was used to aliquot submilligram quantities; 0.25 mg of 4M Walls was mixed with 30 μg of BSA and various amounts of expansins (0.0–2.0 μg) in a total volume of 0.3 mL of 50 mM sodium acetate, pH 4.5. After incubation for 30 min at 25°C, the 4M Walls were sedimented in a microfuge, an aliquot of the supernatant was removed, and 10- and 100-fold dilutions of each incubation were made in 20 mM Tris-HCl, pH 7.0, for subsequent quantitation of unbound expansin by ELISA.

ELISA

For each sample three separate 0.1-mL aliquots were pipetted into the wells of a microtiter plate (EIA/RIA Plate; Costar Corp., Cambridge, MA). A series of dilutions of expansin proteins was also applied to each plate in triplicate. Plates were stored overnight at 4°C. Plates were washed three times in a blocking solution of PBS containing 0.1% BSA and 0.05% Tween 20. Antibodies raised against Ex29 in a New Zealand White rabbit (as described by Li et al., 1993) were incubated in the plates at a dilution of 1:1000 in blocking solution for 1 h at 25°C. After three washes in blocking solution, the plates were incubated with goat anti-rabbit IgG-conjugated alkaline phosphatase (No. A-8025, Sigma) at 1:1000 dilution in blocking solution for 1 h at 25°C. The plates were rinsed three times in blocking solution and once in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5. Plates were then supplied with 0.1 mL/well of 1 mg/mL p-nitrophenyl phosphate in the same buffer. Color development was monitored at 405 nm with a series 750 microplate reader (Cambridge Technologies, Inc., Cambridge, MA).

RESULTS

Expansins Do Not Hydrolyze Hemicelluloses or Pectins

When the hemicellulose fraction from cucumber hypocotyls was incubated with purified expansins at a pH at which they induced wall extension, there was no effect on the molecular mass distribution of the xyloglucans, as assayed by gel permeation chromatography and iodine staining (Fig. 1). Independent tests confirmed that these expansin fractions showed high activity in the wall extension assay (not shown). The total amount of xyloglucan (represented by total area under the curves) did not decrease during these incubations. Endoglycanase activity would be expected to cause a shift to the right in the molecular mass distribution because of a decrease in the average molecular mass of the polymers. Exoglycanase activity would also cause a shift in the distribution to the right and lead to a decrease in the total amount of iodine-stainable material. We confirmed that partial degradation of the hemicellulose fraction with *Trichoderma* cellulase resulted in these changes in the xyloglucan peak (not shown). These results show that expansins do not hydrolyze soluble xyloglucans.

Viscosity assays likewise indicated that expansins lack endoglycanase activity. Although the crude wall protein fraction slowly reduced the viscosity of a hemicellulose solution, this effect disappeared when the more purified
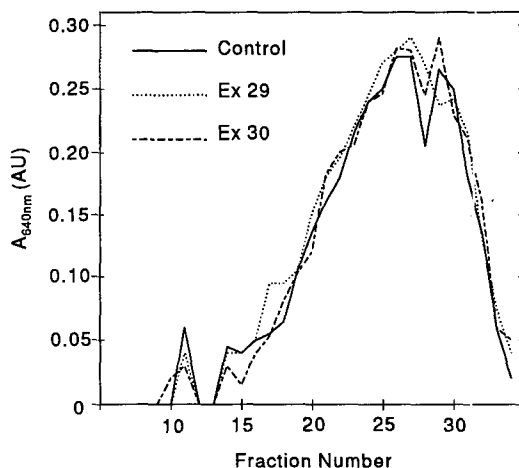

Figure 1. The effects of expansin fractions on the molecular mass distribution of cucumber xyloglucans. Cucumber hemicellulose (5 mg of Hemi-4) was incubated in 1 mL of buffer, pH 4.5, containing no protein (control), 5 μg of active S1 protein (Ex29), or 5 μg of active S2 protein (Ex30), for 4 h at 30°C. Incubation products were separated by gel permeation chromatography on a 75-mL column of Sepharose CL-4B and collected as 2-mL fractions. Aliquots (0.5 mL) from each fraction were assayed for xyloglucan with iodine and A_{640} was recorded. This experiment was carried out three times with similar results. AU, Absorbance units.

expansin fractions were used (Fig. 2A). By comparison, cellulase rapidly lowered the viscosity of the hemicellulose solution, confirming that the hemicellulose was a suitable substrate for glucanases. Taken together, these viscosity data and the molecular mass distribution data show that expansins lack substantial glucanase or xyloglucan endotransglycosylase activity, both of which should lead to detectable changes in both of these assays (Fry et al., 1992; Nishitani and Tominaga, 1992).

Similar viscometric assays were carried out using cucumber pectins (CDTA extracts) as substrates. The C3 protein fraction caused a significant, time-dependent reduction in the viscosity of the pectin solution, indicating that this mixture of proteins retained considerable pectolytic activity (Fig. 2B). The purified expansin fractions (S1 and S2), however, caused no detectable reduction in pectin viscosity above controls, whereas parallel experiments showed them to possess extension-inducing activity similar to the C3 and crude wall protein fractions (data not shown). It appears, therefore, that expansin action does not involve pectin hydrolysis.

Expansins Alter the Stress-Relaxation Spectrum without Progressive Weakening of the Wall

Stress-relaxation experiments were undertaken to examine the effects of expansins on molecular rearrangements in the wall and to test for time-dependent weakening of the walls, which would be expected if expansins possessed hydrolase activity. In these experiments, a hypocotyl wall specimen was held between two clamps and the sample was rapidly extended until a predetermined stress (20 g of tension) in the wall was attained. The wall sample was then
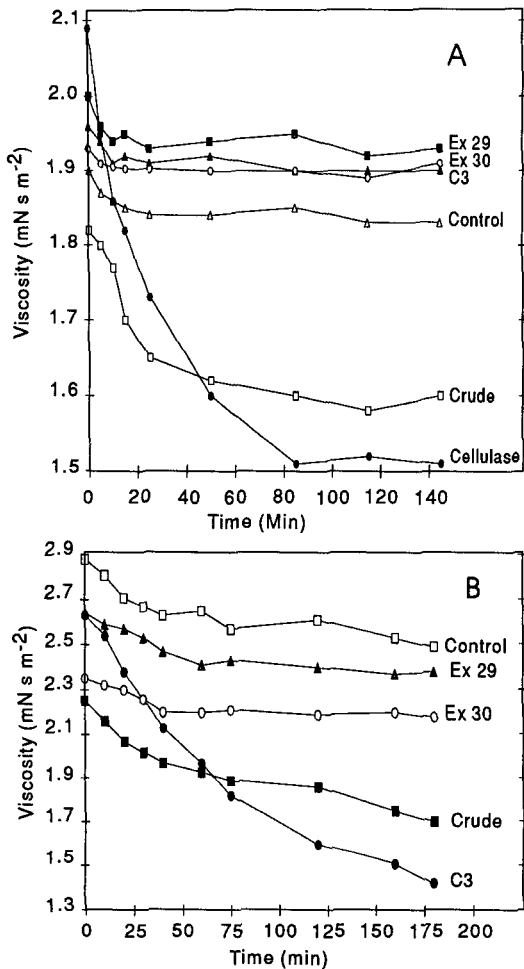
Figure 2. The effects of expansins on the viscosity of hemicellulose (A) and pectin (B) solutions. A, Cucumber hemicelluloses (Hemi-4) were dissolved in 50 mM sodium acetate, pH 4.5, at a concentration of 50 mg/mL. Crude wall protein (2 mg/mL), C3 protein (50 µg/mL), S1 protein (5 µg/mL [Ex 29]), S2 protein (5 µg/mL [Ex 30]), or cellulase (2.5 mg/mL from *T. viride* in volumes of 0.1 mL were added to 0.5 mL of the hemicelluloses. Viscosity of the solutions was measured periodically using a rolling ball viscometer that had been calibrated with Suc solutions of known viscosity. This experiment was carried out three times with similar results. B, Methods as in A, except that cucumber pectins (CDTA-solubilized fraction, 50 mg/mL) were used in place of hemicelluloses. This experiment was repeated four times with similar results.

held at this position (constant strain) and the ensuing relaxation of stress within the wall, due to molecular rearrangements, was detected by a force transducer attached to the lower clamp and recorded by a microcomputer. Data are presented as relaxation rates as a function of log (time). Information regarding the timing of molecular rearrangements within the wall is readily apparent from the relaxation spectra (larger molecular rearrangements take longer than smaller ones and show up later in the spectra; Masuda, 1978).

In Figure 3A the upper curve shows the stress-relaxation spectrum of native (unboiled) cucumber hypocotyl wall
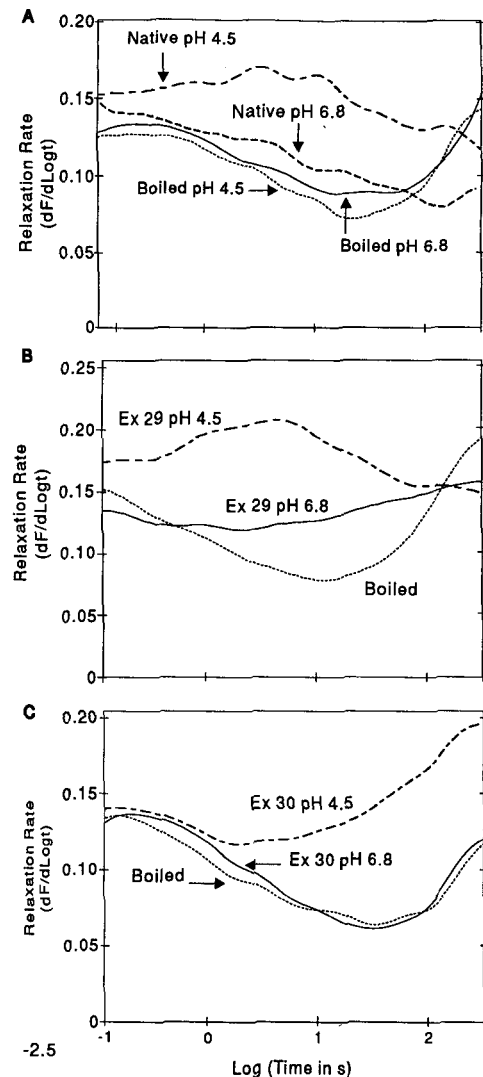
Figure 3. Effects of expansins on the stress relaxation of boiled cucumber hypocotyl walls. A, Native cucumber hypocotyl sections were frozen, thawed, lightly abraded, gently pressed between glass plates (to remove excess fluids and make handling easier), and incubated for 30 min in 50 mM sodium acetate, pH 4.5, at 25°C prior to being subjected to stress relaxation. Boiled tissues received the same treatment, except they were boiled for 15 s in water prior to pressing between glass plates. Wall specimens were kept well hydrated with 50 mM sodium acetate, pH 4.5, or 50 mM Hepes, pH 6.8, during stress-relaxation tests to prevent the tissues from drying out. The pH values designated on the figure are those at which the assay was run. B, Boiled walls were prepared as in A and incubated for 1.5 h in 50 mM sodium acetate prior to stress-relaxation tests. Ex29-treated walls were boiled walls incubated with 5 µg/mL S1 protein at pH 4.5 for 1.5 h at 25°C. At the end of the incubation period, walls labeled "Ex29 pH 4.5" were rinsed in three changes of 50 mM sodium acetate, pH 4.5, during a period of 5 min, whereas walls labeled "Ex29 pH 6.8" were rinsed three times in 50 mM Hepes, pH 6.8, during a period of 5 min to adjust the pH. During stress-relaxation tests tissues were kept hydrated with buffers of the appropriate pH value. C, The treatments were identical with those in B except 5 µg/mL of S2 protein (containing Ex30) replaced S1. Data presented are the means of 10 replicate measurements. All experiments were repeated at least three times with similar results.

specimens at pH 4.5. The rate of relaxation in such experiments was much lower when carried out at pH 6.8. This effect is in agreement with the acid-induced extension seen in these walls. The two lower lines in Figure 3A show that a 15-s treatment in boiling water reduces the rate of stress relaxation over the time range of 0.5 to 300 s. Such heat treatment also inactivates the expansin-mediated extension of these walls (Cosgrove, 1989; McQueen-Mason et al., 1992). Interestingly, there is little or no effect of pH on the stress-relaxation spectra of heat-treated walls. This suggests that the acid-enhanced stress relaxation in these walls is not a direct physicochemical effect of pH on wall structure but is due to a heat-labile activity.

When boiled walls were reconstituted with the S1 fraction containing Ex29, the rate of relaxation was greatly enhanced over boiled control walls (Fig. 3B). Thus, addition of expansin restores most, if not all, of the stress relaxation lost by boiling the walls. When boiled wall specimens were reconstituted with Ex29 at pH 4.5 but assayed at pH 6.8, they showed substantially reduced rates of relaxation compared with those assayed at pH 4.5. This is in agreement with the reported pH sensitivity of expansin activity (McQueen-Mason et al., 1992). A similar phenomenon was seen when boiled walls were reconstituted with the S2 fraction, containing Ex30 (Fig. 3C). Both sets of walls were preincubated with Ex30 at pH 4.5; yet the rate of relaxation is much lower in those assayed at pH 6.8 than those at pH 4.5. We conclude that expansins are responsible for the component of the stress relaxation of native walls that is pH dependent and heat labile.

The relaxation spectra of walls reconstituted with Ex29 and Ex30 are rather different. The effects of Ex29 at pH 4.5 were most evident in the middle of the spectrum (between 1 and 30 s), where the effects of Ex30 just become apparent. In the later part of the relaxation spectrum (>100 s), Ex29 has little or no effect, perhaps even depressing the rate of relaxation, whereas at this point the walls treated with Ex30 show enhanced relaxation. It is also apparent that Ex29 caused considerable elevations in the rate of relaxation at pH 6.8, whereas Ex30 was ineffective at this pH (Fig. 3C).

These observations indicate that Ex29 and Ex30 may act on different components of the wall because Ex30 incubations affect the slower parts of the spectrum, whereas Ex29 affects somewhat faster elements of the relaxation spectrum. It should be noted that when walls were incubated with a combination of Ex29 and Ex30, their effects were additive rather than synergistic (data not shown).

These data also show that the expansins need to be in an active conformation (pH 4.5, not pH 6.8) during stress relaxation for their effects to be apparent. This shows that expansins do not irreversibly weaken the wall during the prolonged (nonstressed) preincubation period in pH 4.5 buffer. To investigate this phenomenon further, we examined the effects of short- and long-term incubations with expansins. Figure 4A shows that there was little or no difference in the rate of relaxation of boiled walls incubated with Ex29 for 10 min or for 90 min. Similar results were obtained using Ex30 (data not shown). Thus, the walls did
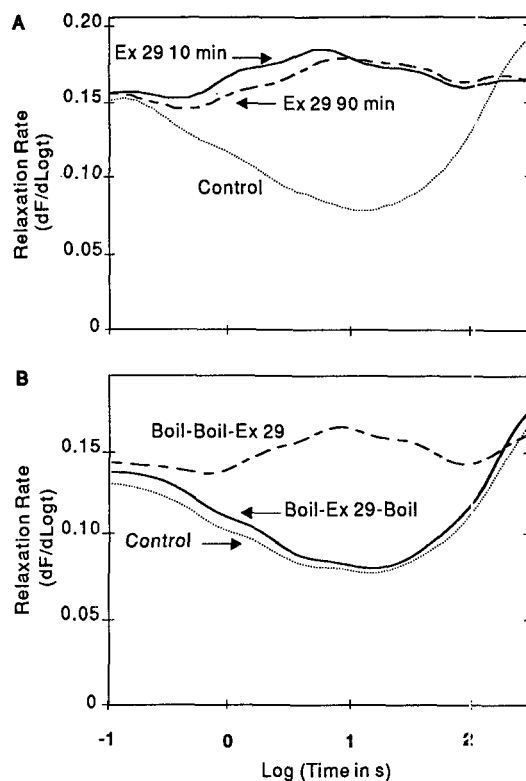

Figure 4. Time dependence of expansin action on stress relaxation of boiled cucumber hypocotyl walls. A, Boiled wall specimens were prepared as in Figure 3A; control specimens were incubated for 90 min in 50 mM sodium acetate, pH 4.5, at 25°C and then stored frozen prior to stress-relaxation tests. Boiled wall specimens were incubated with 5 μg/mL of S1 protein (containing Ex29), pH 4.5, at 25°C for either 10 or 90 min and then stored frozen prior to stress-relaxation tests. B, Boiled wall specimens were given one of three treatments: The first group was incubated with S1 protein (containing Ex29) at pH 4.5 for 1 h at 25°C and then boiled for 15 s in water to inactivate the proteins. The second group of boiled walls were boiled a second time for 15 s in water and then incubated in S1 protein at pH 4.5 for 1 h at 25°C. Control boiled wall specimens were incubated in 50 mM sodium acetate, pH 4.5, for 1 h at 25°C and then boiled for 15 s in water. Stress-relaxation spectra represent the mean values of 10 replicate measurements in each case. Similar results were obtained in three independent experiments.

not become mechanically weaker during the intervening 80 min. This suggests two possible scenarios: either the reaction had gone to completion in 10 min (unlikely because these proteins are capable of catalyzing steady rates of extension for hours [McQueen-Mason et al., 1992]) or expansins lack wall-weakening activity in the absence of wall tension. These results are informative because we would expect a time-dependent weakening of the wall upon incubation with these proteins if the expansins were hydrolyzing polymers within the wall.

To pursue this point further, we investigated the effect of inactivating the expansins at the end of an incubation, prior to the stress-relaxation measurement. In Figure 4B, wall specimens were boiled for 15 s in water to inactivate them. Wall specimens were then divided into three groups. One

group was immediately boiled again for 15 s and then incubated with Ex29 at pH 4.5. The second group was incubated with Ex29 and then boiled for 15 s to inactivate the expansins. The control group was incubated in pH 4.5 buffer, without added proteins, and again boiled at the end of the incubation. The wall specimens that were boiled after incubation with Ex29 exhibited a relaxation profile typical for inactivated wall specimens. The altered relaxation upon incubation with Ex29 was thus fully reversible by heat inactivation. Walls subjected to two rounds of boiling followed by Ex29 incubation show the enhanced relaxation spectra of a typical Ex29 incubation. Similar results were obtained using Ex30 (data not shown). We conclude that expansins do not progressively weaken the wall in a time-dependent fashion and that their effects on wall relaxation are fully reversible by heat inactivation.

Expansins Bind to Cellulosic Wall Residues in Vitro

Binding studies were undertaken to determine the relative affinity of expansins for various cell wall fractions from cucumber hypocotyls. The assumption in these experiments was that expansins have high affinity for the substrate on which they act to cause extension. Identification of this substrate is of key importance in understanding the mechanism of expansin action. In these studies C3 proteins (containing both Ex29 and Ex30) were mixed with cell wall or solubilized wall polysaccharide fractions prior to the extension assay. We assumed that the wall fractions (or polysaccharides) would compete with the extending wall for the binding of expansins and hence lower the expansin activity in the solution. Moreover, we expected that the penetration of exogenous expansin into the wall specimen would be greatly hindered if the protein was bound to a large polysaccharide outside the specimen.

For these studies cucumber hypocotyl cell walls were sequentially extracted to solubilize matrix components away from the cellulosic fibers of the walls (see Fig. 5 for diagram). Where possible the soluble components were retained and used in binding experiments, as were the insoluble wall residues after each step. Four different fractions of matrix polymers solubilized from cucumber cell walls were used in these binding experiments. These were wall proteins (released by 1 M NaCl), pectins (released by 50 mM CDTA), Hemi-4 (hemicelluloses released by 4 M NaOH), and Hemi-6 (hemicelluloses released by 6 M NaOH). As shown in Figure 6A, none of these solubilized fractions significantly bound exogenously applied expansin. These results indicate that expansins have little or no affinity for solubilized matrix polysaccharides and proteins.

In contrast, when similar experiments were carried out using the insoluble wall residues, most of the expansin activity was bound to the residues (Fig. 6B). This binding was specific, because when 1 mg/mL BSA was included with the expansin solution there was no appreciable difference to binding in the absence of BSA (data not shown). When walls from which soluble proteins were removed (by NaCl and PAW extraction) were mixed with the expansin solution, about 75% of expansin activity bound to this
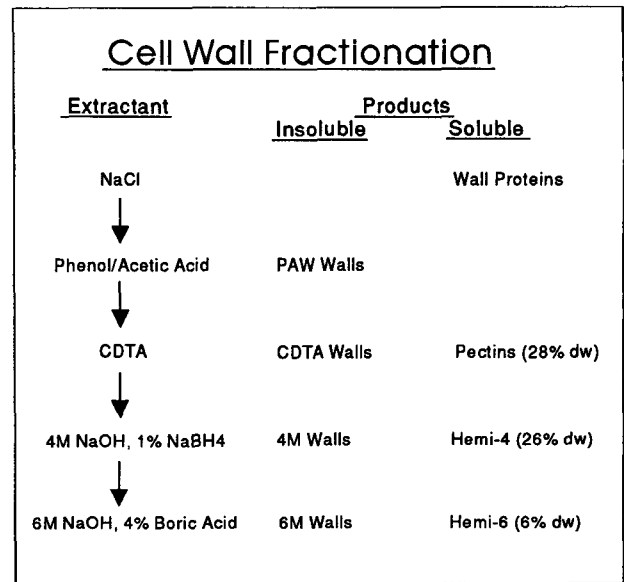
Cell Wall Fractionation

Extractant	Products	
	Insoluble	Soluble
NaCl		Wall Proteins
↓		
Phenol/Acetic Acid	PAW Walls	
↓		
CDTA	CDTA Walls	Pectins (28% dw)
↓		
4M NaOH, 1% NaBH4	4M Walls	Hemi-4 (26% dw)
↓		
6M NaOH, 4% Boric Acid	6M Walls	Hemi-6 (6% dw)

Figure 5. Diagram of cell wall fractionation procedures. Growing cucumber hypocotyl cell walls were fractionated by extraction in 1 M NaCl and then in PAW to remove soluble proteins. Salt-extracted proteins were desalted, boiled for 5 min, and dried under vacuum (Wall Proteins). A sample of the PAW-extracted walls was washed and dried for later use (PAW Walls). De-proteinized walls were extracted in 50 mM CDTA to remove pectins, which represented about 28% of the dry weight (dw) of the starting material. The extract was desalted and dried (Pectins), and a sample of walls was washed, dried, and retained (CDTA Walls). CDTA-extracted walls were extracted in 4 M NaOH, 1% NaBH$_4$ to remove hemicelluloses; the extract was neutralized, desalted, and dried. This fraction accounted for about 26% of wall dry weight and was designated the Hemi-4 fraction; a sample of walls was washed, dried, and retained and designated 4M Walls. The remaining walls were extracted in 6 M NaOH, 4% boric acid to remove more tightly associated hemicelluloses representing 6% of wall dry weight; the extract was neutralized, desalted, and dried (Hemi-6), and the wall residues were washed and dried (6M Walls).

material. Subsequent extraction of pectins from these walls increased the binding activity of these wall residues (86% of activity bound), suggesting that pectins do not bind expansins. Removal of most of the hemicelluloses by extraction with 4 M NaOH further increased the binding activity of the wall residues (95% activity bound). This result suggests that the bulk of the hemicelluloses are unimportant for expansin binding; however, it may be pertinent that 4 M NaOH causes swelling of native cellulose to form the less crystalline form cellulose II, which contains more extensive amorphous regions than the native form. This swelling could be important for expansin binding and will be discussed later. Extraction of 4M Walls with a stronger alkaline solution (6 M NaOH, 4% boric acid) diminished very slightly the binding affinity of the walls (6M Walls) for expansins. Although this effect was not statistically significant, we found that three successive extractions of 4M Walls in 6 M NaOH, 4% boric acid dramatically lowered expansin binding (discussed in the next section). This treatment removes more tightly bound hemicelluloses
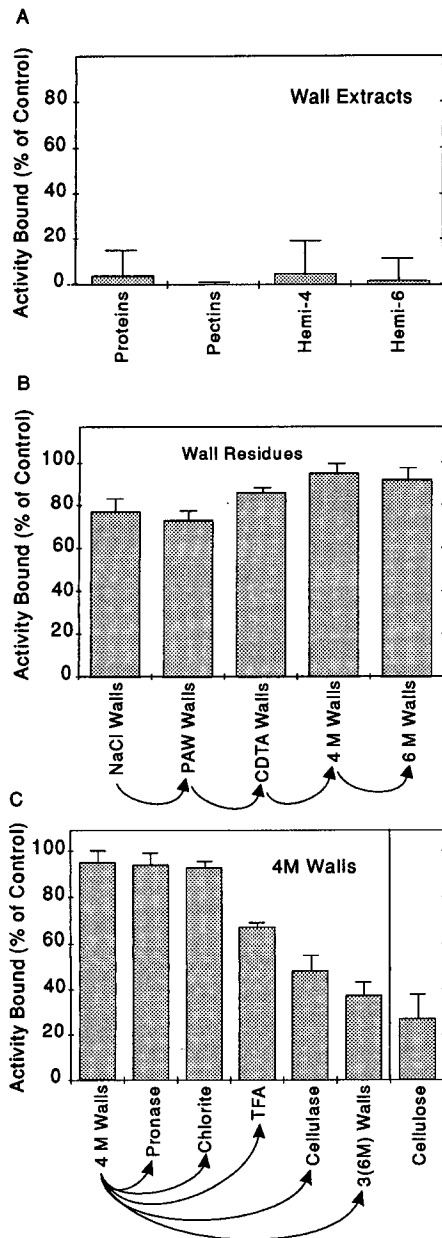
but could also lead to "peeling" (degradation) of both the hemicelluloses and exposed β-(1-4)-glucans in amorphous regions of the cellulose. The fact that the soluble Hemi-6 polymers do not bind out expansins (Fig. 6A) indicates that these polymers have little affinity for expansins, at least not in the form of soluble polymers.

We conclude from these experiments that expansins do not bind to soluble matrix polymers, but they do associate with the insoluble wall fraction that is composed of cellulose and some tightly associated polymers.

Expansin Binding to Cellulosic Wall Residues Is Diminished by Treatments That Remove Noncrystalline Regions

To determine which part of this alkaline-insoluble fraction was important for expansin binding, further extractions and digestions of 4M Walls were carried out. 4M Walls might contain insoluble structural proteins covalently linked to the walls that could be important for the binding of expansins. Prolonged digestion of 4M Walls with Pronase, a nonspecific mix of proteolytic enzymes, had no effect on the binding affinity (Fig. 6C), indicating that expansins do not associate with structural proteins. 4M Walls were also subjected to a hot chlorite treatment. This treatment should destroy phenolic linkages in the walls and thus solubilize polymers such as lignins or structural proteins that might be tied to the walls by such linkages. This treatment did not decrease the binding affinity of 4M Walls, suggesting that expansins do not bind to polymers held in the wall by such associations.

In contrast, partial digestion in 2 M TFA at 120°C significantly decreased the binding affinity of 4M Walls for expansins; treated walls bound only about 70% of the activity compared to 95% for the 4M Walls. This treatment hydrolyzes noncrystalline polysaccharide polymers, including hemicelluloses and amorphous regions of cellulose.

After cellulase treatment the 4M wall residues bound only about 57% of the activity, a marked reduction in binding affinity. The effects of this digestion on wall structure are probably similar to those of TFA. Commercial

Figure 6. Binding of expansin activity to soluble and insoluble cucumber cell wall fractions. A, C3 protein (40 μg/mL) was mixed with 10 mg/mL soluble extracts from cucumber hypocotyl cell walls in 50 mM sodium acetate, pH 4.5. Extracts were obtained by the sequential extraction procedure described in Figure 5. Proteins are from NaCl extracts; these proteins were boiled before mixing with expansins (to eliminate expansin activity in this extract). The pectin and hemicellulose fractions (Hemi-4 and Hemi-6) were all dialyzed and dried before use. The expansin/extract mixture was then applied to heat-inactivated cucumber hypocotyl sections in an extension assay. Hypocotyls were extended in 50 mM sodium acetate, pH 4.5, for 30 min before addition of the mix. Extension activity was calculated as extension rate after addition of the expansin/extract mix minus extension rate before addition. Control (expansin without extracts) extension activity was 3.55 μm/min with an SE of 0.04 ($n = 28$). Activity bound was calculated as the percentage of control activity lost by addition of the wall fraction to the expansins solution. All experiments were repeated three times with similar results. B, Exper-

iments were essentially the same as in A, except that the insoluble wall residues that remained after each sequential extraction were used in place of the soluble extracts. The fractions used were as follows: walls extracted in PAW (PAW Walls), walls subsequently extracted in 4 M NaOH (4M Walls), walls subsequently extracted in 6 M NaOH (6M Walls). Activities are calculated and expressed as in A. Results are means and SE of four measurements. C, The same binding of activity assay was used as in A and B except that the binding material consisted of 4M Walls that were subjected to a further series of separate digestions. The binding fractions used were as follows: 4M Walls digested with proteolytic enzymes (pronase), 4M Walls treated with a hot chlorite solution (Chlorite), 4M Walls treated at 120°C in 2 M TFA (TFA), 4M Walls digested with cellulase (Cellulase), 4M Walls give three sequential extractions n 6 M NaOH [3(6M) Walls], and finally purified cotton cellulose was also used as a binding fraction (Cellulose). Results are means and SE of four measurements; overall experiments were repeated three times with similar results.

cellulase preparations typically include many other enzymes in addition to cellulases and will digest most polysaccharides in cell walls. These enzymes are not efficient in hydrolyzing crystalline cellulose but prefer the solvated β-(1–4)-chains in amorphous regions of the fibrils and will have the effect of cleaning the fibrils of hemicelluloses and noncrystalline cellulose regions.

When 4M Walls were given three successive 24-h washes in 6 M NaOH, 4% boric acid [3(6M)Walls], the binding activity was dramatically lowered. This treatment is also likely to strip off tightly associated hemicelluloses as well as cause peeling of hemicelluloses and amorphous regions of cellulose.

Finally, when commercially available celluloses were used in similar binding studies they displayed low affinity for expansins. In the example shown in Figure 6C, fibrous cellulose bound only about 30% of the activity in the C3 fraction. Similar results were obtained using other commercially available cotton celluloses.

In summary, the data in Figure 6 indicate that the binding affinity of wall residues for expansins is a function of noncrystalline polysaccharides strongly associated with cellulose microfibrils.

Coating Cellulose Fibers with Soluble Polysaccharides Increases Expansin Binding

We conjectured that, if expansins indeed bind to noncrystalline polysaccharides on the microfibril surface, then it should be possible to increase the binding activity of pure cellulose or 3(6M) Walls, both of which have low affinity, by coating them with polysaccharides. In our experience, hemicelluloses (in particular xyloglucans) do not bind effectively to cellulose in solution. Binding is greatly enhanced when the xyloglucans and cellulose are dried together at 80°C. This might be due to two factors: first, drying the samples will enhance hydrogen bonding between the polymer systems; second, high temperatures may cause swelling of the cellulose microfibrils, opening spaces between β-glucan chains and allowing the integration of hemicelluloses into the cellulose microfibrils. This model has been used to explain the increased efficiency of the incorporation of xyloglucans into cellulose at high temperature (Hayashi et al., 1994). Because of these considerations, we coated cellulose with soluble polysaccharides by drying the mixtures together at 80°C to optimize coating.

In Figure 7A the effects of coating 3(6M) Walls are shown. The uncoated walls removed only about 42% of the expansin activity. Binding of some soluble polysaccharides to these wall residues significantly increased their affinity to expansins, whereas other polymers had little effect. Pectins, xyloglucans, and xylans had little or no effect on expansin binding, whereas the cucumber Hemi-4 and Hemi-6 fractions as well as the barley β-glucan markedly increased the amount of expansin activity bound out by the wall fractions.

Similar experiments were performed to see whether coating cellulose with the same set of polysaccharides would increase their affinity for expansins. The results appear in Figure 7B and are similar to those obtained with
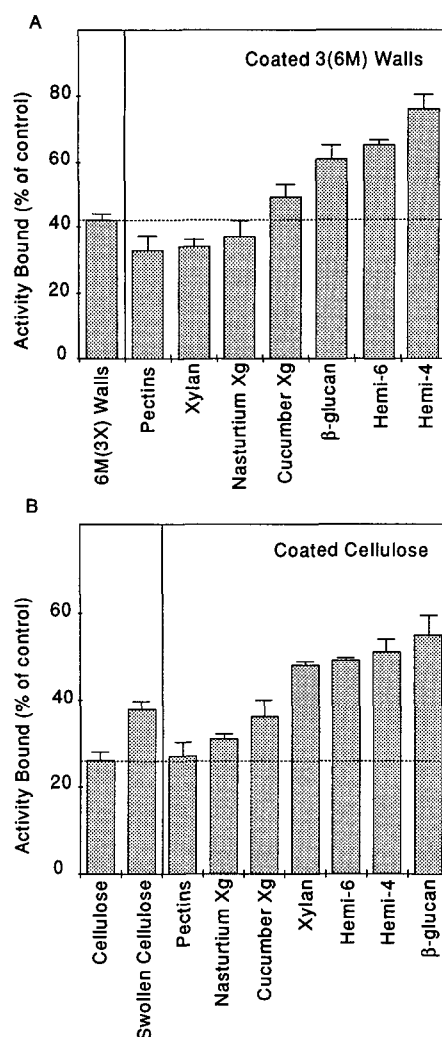

Figure 7. The effect of polysaccharide coating on the binding of expansin activity to NaOH-extracted walls or commercially available cellulose. A, Extracted cucumber walls 3(6 M) with lowered expansin-binding activity (Fig. 6C) were coated with a range of soluble polysaccharides from cucumber cell walls or from other sources and tested for expansin-binding ability. The polysaccharides were pectins from cucumber (Pectins), hemicelluloses from cucumber (Hemi-4 and Hemi-6), xyloglucans from cucumber (Cucumber Xg) or nasturtium seed (Nasturtium Xg), xylan from oat spelts (Xylan), and β-glucan from barley (β-glucan). Results are means and SE of four assays; overall experiments were repeated twice with similar results. B, Experimental setup was identical with those in A, except for two features: first, cellulose (Cellulose Fibrous Medium, Sigma) was used instead of 3(6 M) Walls for coating; second, the binding activity of cellulose that had been swollen in 4 M NaOH was also tested (Swollen Cellulose). Results are means and SE of four measurements; overall experiments were repeated twice. The horizontal dotted lines mark the binding values for uncoated 6 M(3X) Walls (A) and uncoated cellulose fibers (B).

3(6M) walls; Hemi-4, Hemi-6, and β-glucan polymers were most effective in increasing expansin binding to the cellulose fibers, whereas pectins and xyloglucans were not effective. Note that treatment of cellulose with 4 M NaOH (swollen cellulose) was also somewhat effective in increas-

ing expansin binding. This treatment is reported to cause swelling of cellulose, leading to a decrease in the crystallinity of the fibers (Zeronian, 1985) and hence an increase in the amount of solvated β-glucans.

It was possible that the effect of recoating the cellulosic wall residues with polysaccharides may be nonspecific in terms of the polysaccharide used and that differences in expansin-binding activity merely reflected the amount of polymer bound to the cellulose. This does not appear to be the case. The amount of soluble polysaccharide bound to the wall residues was calculated from the increase in dry weight of insoluble matter after binding (Table I). The Hemi-4-coated 3(6M) walls were clearly the most effective for expansin binding (Fig. 8A), whereas they absorbed only a modest amount of polysaccharide (16% of their dry weight). In contrast, more of the nasturtium xyloglucan bound to the 3(6M) walls (28% increase in dry weight), but this did not enhance expansin binding above that of uncoated control 3(6M) walls. This suggests that binding is not merely a function of the amount of coating on the cellulose but involves a specific type of polymer.

In view of the conventional model of wall in which xyloglucan tether microfibrils together, it is notable that a xyloglucan coating was rather ineffective in raising the binding of expansins. For instance, in Figure 7A compare the binding values for the uncoated 6 M(3X) Walls (42%) with cucumber xyloglucan-coated walls (48%) and Hemi-4-coated walls (76%). The xyloglucans are a subfraction purified out of the Hemi-4 fraction, and their poor effectiveness indicates that the specific binding activity of the Hemi-4 fraction is not due to xyloglucans alone but requires some other hemicellulosic polysaccharide. These results are also consistent with the data in Figure 6, in which xyloglucan extraction (with 4 M NaOH) increases the specific binding activity of the wall.

In summary, the data in Figures 6 and 7 show that expansins bind to cellulosic residues in a manner that is dependent on the presence of noncrystalline regions on the surface of the cellulose. Such regions appear to represent junctions between cellulose microfibrils and one or more minor components of the matrix hemicelluloses.

Expansin Binding to Walls Is Tight, Saturable, and Related to Wall Extension Activity

Experiments were carried out to quantify the binding of expansins to 4M Walls. Figure 8 shows a binding curve for Ex29 to 4M Walls. For these measurements varying concentrations of expansins were mixed with constant
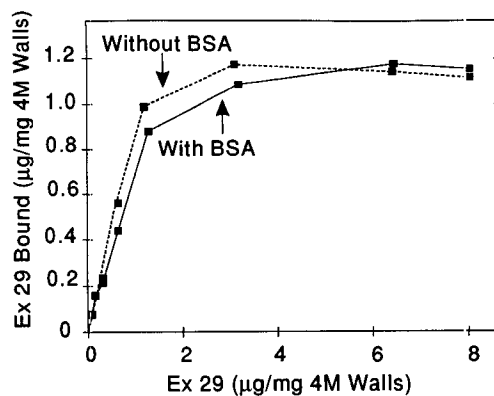

Figure 8. Binding of expansins to 4 M Walls. Varying concentrations of Ex29 or Ex30 were mixed with a constant concentration of 4 M Walls with or without the inclusion of 100 μg/mL BSA to block nonspecific binding sites. After equilibration, aliquots of the free solution were removed and the amount of unbound expansin was determined by ELISA. Results shown are for Ex29; similar results were obtained with Ex30. Experiments were repeated twice with similar results.

amounts of 4M walls and allowed to equilibrate. An aliquot of the free solution was then removed and the amount of unbound expansin was quantified in an ELISA using antibodies raised against Ex29. Binding of expansins to 4M Walls saturated at about 1.2 μg of expansin/mg of 4M Walls, a ratio of about 1:800. Inclusion of BSA in the binding experiments (at 20 times the highest concentration of expansins used) made very little difference to the binding of expansins, shifting the curve slightly but not affecting saturation values (Fig. 8, dashed line), indicating that expansin binding is specific. Binding of Ex30 was very similar to that for Ex29; Ex30 saturated 4M Walls at about 1.4 μg of Ex30/mg of 4 M Walls, and there was little effect of BSA on binding (not shown).

To assess the relationship between expansin binding and expansin activity in native cell walls, we extracted the walls with various solutions and compared the amount of expansin protein extracted (Fig. 9A) with the amount of expansin activity remaining in the walls (Fig. 9B). Extraction with buffer alone did not release detectable amounts of expansin protein and the walls had high extension activity (as assayed by extension induced by pH 4.5 buffer; McQueen-Mason et al., 1992). Extraction with 0.2% Triton X-100 also failed to extract expansins or reduce expansin activity in the walls. When walls were extracted with either 1 M NaCl

Table I. *Efficiency of coating 3(6M) Walls or cellulose with soluble polysaccharides*

Commercially available cellulose or base-extracted cucumber walls [3(6M) Walls] were mixed, in aqueous suspension, with a variety of soluble polysaccharides in proportions of 2 parts insoluble walls or cellulose to 1 part soluble polysaccharide. The mixture was dried to facilitate coating and then extensively washed to remove unincorporated polymers. Insoluble matter was dried and weighed and the percentage of polymer bound calculated from the increase in dry weight. Experiments were performed twice with similar results. Xg, Xyloglucan.

Polysaccharide	Pectins	Hemi-4	Hemi-6	Cucumber Xg	Nasturtium Xg	Xylan	β-Glucan
% Bound to 3(6 M) Walls	12	16	14	24	28	18	12
% Bound to cellulose	12	15	12	22	24	7	8

of walls and completely eliminated expansin activity in the walls.

This amount (193 ng/mg) is one-sixth the amount of expansin required to saturate 1 mg of 4M Walls. The difference in these two values might be explained in a number of ways. First, 4M Walls are a subfraction of native walls and account for about 30% of the dry weight of unextracted walls. If we correct for this, we can calculate a saturation of about 360 ng/mg of native walls (we could not assess binding of expansins to native walls directly because of interference from native expansins). Second, perhaps not all available sites in the walls are occupied by expansins. Third, preparation of 4M Walls involves extraction with 4 M NaOH, a treatment that causes cellulose swelling and increased expansin binding (Fig. 8B).

To compare expansin binding in native walls with binding in vitro, we used the same set of solutions to extract expansins from 4M Walls preincubated with expansins. Only SDS proved very efficient in this task, removing about 92% of the expansins from the wall (Fig. 10). Guanidine HCl (1 M) removed only 8% of the antigen, whereas NaCl, urea, and Triton X-100 removed no detectable quantities of expansin. These results indicate that expansin binding to 4M Walls is similar in nature to expansin binding to the active site in native cell walls.

DISCUSSION

Our results point to three major conclusions about the action of expansins on the plant wall: (a) expansins lack

Figure 9. Extraction of expansins and removal of expansin activity by various treatments. A, Native cucumber hypocotyls were extracted for 1 h in different solutions made up in 50 mM sodium acetate, pH 4.5. After extraction the amount of expansin in the solution was calculated using ELISAs. B, Walls were extracted with the same set of solutions as were used in A. After the walls were rinsed to remove the extraction solution, they were assayed for expansin activity in an extensometer. Activity was calculated as the rate of extension at pH 4.5 minus the rate of extension at pH 6.8. Overall experiments were repeated twice with similar results. Data are means and SE of three ELISA measurements and four extensometer measurements. After extraction and assay the hypocotyl sections were weighed, and the average dry weight of a 1-cm hypocotyl section was 0.39 mg.

or 1 M urea, small amounts of expansin protein were released, but this had little effect on expansin activity in the walls. In contrast, extraction with 1 M guanidine HCl extracted more expansin and eliminated about 85% of expansin activity from the walls (probably partly by denaturation). The most effective extractant was SDS, which released 193 ng of expansin protein from 1 mg (dry weight)
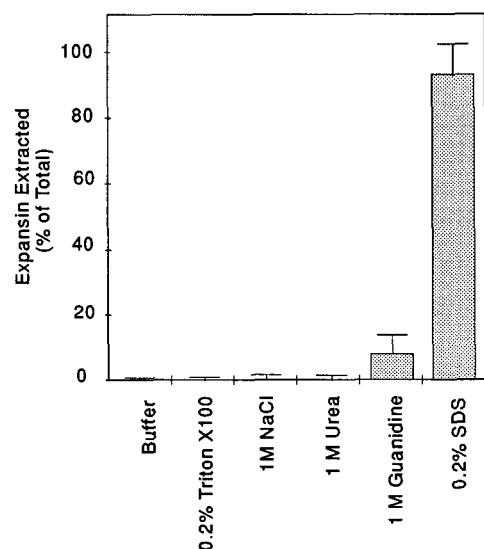
Figure 10. Extraction of expansin that had been bound to 4M Walls. Six 10-mg samples of 4M Walls were incubated with a solution containing 2 μg of expansins in 2 mL of 50 mM sodium acetate, pH 4.5. The wall suspensions were then centrifuged onto a filter (Millipore Ultrafree-MC 10,000 NMWL) to remove the free solution and washed three times on the filters with 50 mM sodium acetate, pH 4.5. The 4M Wall samples were then incubated for 30 min with the same extraction solutions as were used in Figure 9. After separation from the filtration, the extracts were quantified for expansins by ELISA. Results are means and SE of three measurements. Experiments were repeated twice with similar results.

endoglycanase activity, (b) expansin activity is a major determinant of stress relaxation in isolated walls, and (c) the site of expansin binding, and probably its site of action, is the noncrystalline glycan coating on the surface of the cellulose microfibril. Each of these points is discussed in detail below.

Expansins Do Not Hydrolyze Matrix Polysaccharides

Cellulose microfibrils within a growing plant cell wall are believed to be embedded in a pectic gel and coated by hemicelluloses long enough to span neighboring microfibrils, serving to anchor them together (McCann et al., 1990). The adhesion between microfibrils and hemicelluloses is thought to result from extensive hydrogen bonding between them (reviewed by Hayashi, 1989). Many reports have indicated an important role for xyloglucans (the major hemicellulose in dicot plants) in cell wall extension in dicot plants. Labavitch and Ray (1974) showed that the xyloglucan fraction of the cell wall was specifically turned over during auxin-induced growth in pea epicotyls. Auxin-induced growth of azuki bean epicotyls was blocked by the application of antibodies raised against xyloglucans (Hoson et al., 1991) and also by incubation with Fuc-binding lectins, which bind to the terminal Fuc of xyloglucan side chains (Hoson and Masuda, 1991). Talbott and Ray (1992) reported remarkable changes in xyloglucan size distribution following excision of pea epicotyls and application of auxin (see also Nishitani and Masuda, 1983). In grasses, which lack appreciable xyloglucan, a β-(1–3,1–4)-glucan appears to play a similar role to that of xyloglucan in dicots (Carpita and Gibeaut, 1993).

In contrast to the picture above, our data show that expansins do not hydrolyze the major components of the matrix. Purified expansins did not reduce the viscosity of pectins and hemicelluloses extracted from the cucumber wall; nor did they alter the molecular size distribution of xyloglucans. Both of these assays are sensitive indicators of endo-type glycanase activity; therefore, we conclude that expansins possess negligible endohydrolase activity against the major polysaccharides of the wall matrix. If expansins hydrolyzed a minor component of the matrix with great specificity, such action might be missed in our assays, in which total cucumber pectins and hemicelluloses were used. However, this possibility is unlikely in view of the fully reversible effects of expansins on the stress-relaxation spectra of walls. Moreover, the effects of expansins on stress relaxation did not increase in a time-dependent fashion, as might be expected from the progressive weakening of a hydrolytic process.

Previous work showed that expansins lack exoglycanase activity and xyloglucan endotransglycosylase activity (McQueen-Mason et al., 1992, 1993). Taken together, our results lead us to conclude that expansins do not hydrolyze xyloglucan or other major components of the matrix.

Expansins Enhance Wall Stress Relaxation

Stress relaxation of isolated walls has been used in previous work to examine the structural rearrangements of the wall that occur after treatment of plant tissues with growth hormones and related agents (Masuda, 1978; Hoson and Masuda, 1991). It is a remarkable fact that the growth hormone auxin can stimulate the rate of cell expansion by a factor of 5 or more, and yet it has only a modest effect on the stress-relaxation behavior of the isolated oat coleoptile walls (Yamamoto and Masuda, 1971; Masuda, 1978). The principal effect is reported to be a slight extension of the relaxation spectrum to slightly faster times, e.g. the minimum relaxation time shifts from 19 to 15 ms (see table 4 in Masuda, 1978). This modest effect has been interpreted as a result of xyloglucan hydrolysis.

In contrast, we found that expansins enhance wall relaxation over a broad range of times. Moreover, they alter the stress-relaxation spectrum of walls in a manner that appears to account for both the pH dependence and the heat inactivation of wall relaxation processes. These are surprising results because they mean that pH alters wall relaxation principally via expansin action and that other heat-labile wall proteins (cellulases, pectinases, peroxidases, etc.) exert a negligible effect on wall relaxation, at least in the time range measured in our assays (0–5 min).

Because Ex29 and Ex30 showed different pH sensitivities and enhanced stress relaxation in different parts of the spectrum, we suggest that the two forms of expansin function in a slightly different manner. Additionally, both the reversibility and the lack of progressive action of expansins on the stress-relaxation behavior of walls are curious results that suggest to us that expansins act only when the walls are under tension.

The comparison between the large effects of expansins on wall relaxation and the small effects of auxin, noted above, might suggest that auxin does not act via a modulation of expansin action. However, this conclusion is not warranted because during the standard procedure used in the Masuda laboratory segments are boiled in methanol and then rehydrated, evidently in water at uncontrolled pH. Methanol boiling does not irreversibly inactivate cucumber expansins (Cosgrove, 1989; McQueen-Mason et al., 1992); therefore, expansins may still be active in the samples prepared by this procedure. However, if the wall pH after rehydration is not in the range of high expansin activity, it is likely that differences in expansin activity would be missed. Because this appears to be the case in most of the published work concerning wall relaxation and auxin action, no firm conclusions can be reached regarding auxin and expansin action from most of the data in the literature. Yamamoto et al. (1974) did carry out some stress-relaxation measurements in pH buffers and found that pea epicotyl walls incubated in pH 4.5 buffer relaxed faster than walls incubated in pH 6.5 buffer (see their table 3, where the shorter Tm values indicate faster relaxation). This observation is consistent with our results, but an exact comparison is not possible because they did not report full spectra but only some fitted parameters, which greatly abstract the spectra. Interestingly, some of the auxin-pretreated segments did show faster relaxation (reported as shorter Tm) when the walls were assayed at pH 4.5, but this issue will need closer study for firm conclusions.

To summarize this section, our results indicate that expansins are the major determinant of the heat-labile and pH-dependent components of stress relaxation in cucumber walls. The reversibility of expansin action on stress relaxation suggests a noncovalent alteration of the wall's load-bearing network(s). The binding studies (next) give us clues about the site of expansin action.

Expansins Bind to the Interface between the Microfibrils and the Matrix

Our binding studies showed that expansins associate strongly with the noncrystalline regions of cellulose and probably with some polymers tightly associated with the surface of the microfibril. Binding to pure cellulose fibers and to "cleaned" walls (i.e. walls extracted of matrix polysaccharides and then further digested with TFA or cellulase) was rather weak but could be considerably enhanced by coating the cellulose or wall residues with specific hemicelluloses. Because elution of expansins from native walls showed the same sensitivity to various extractants as did the removal of expansin activity (Figs. 9 and 10), the binding site is probably also the site of expansin action. This binding site appears from our results to be at the interface between cellulose microfibrils and matrix polysaccharides.

Curiously, xyloglucans were not very effective as a binding material, either in the soluble form or as a coating on cellulose or extracted cucumber walls (Figs. 6 and 7). This suggests that a hemicellulosic component other than xyloglucan may be more important as a cellulose coat and tether upon which expansins act. This conclusion, if proved true, would require a revision of the conventional model of the wall, in which xyloglucans serve as load-bearing tethers between cellulose microfibrils (Fry, 1989; Hayashi, 1989; Carpita and Gibeaut, 1993).

In view of the fact that we routinely extract expansins from native cucumber walls with 1 M NaCl, we were surprised to find that most of the expansin remained bound to the wall after such extraction and could be extracted only with SDS (Fig. 10). We have also confirmed this result with western blots of the protein extracted from native walls (data not shown). It appears that 1 M NaCl extracts less than 20% of the expansins from native walls; unfortunately, expansins extracted with SDS possess little activity, evidently because they are denatured.

How Do Expansins Promote Wall Extension?

Although we find no evidence that expansins possess hydrolase activity, several of our observations indicate that expansins act catalytically on the wall. In growing cucumber cell walls, the ratio of SDS-extractable expansins to dry weight of cell walls is very low (193 ng/mg, or about 1:5000). Furthermore, isolated walls (native or reconstituted with expansins) can extend for several hours, leading to greater than 40% increase in length, in the absence of the synthesis of new wall components (McQueen-Mason et al., 1992). These observations demonstrate that expansins are not simply stoichiometric additions to the wall during

expansion. However, the stress-relaxation results suggest that expansins do not alter the covalent structure of the wall. Rather, expansins behave as if they either induce a reversible phase change in a selective component of the wall or act only when the wall is in tension (or both).

As an analogy to explain our results, we suggest that expansins act as a sort of biochemical grease. Rather than cleaving covalent bonds, expansins might facilitate polymer slippage under stress by disrupting noncovalent binding between wall polymers, e.g. hydrogen bonding of matrix polysaccharides to cellulose microfibrils. Such a mechanism is attractive because it would produce little or no molecular rearrangements unless the walls were under stress. This mechanism would not lead to the degradation of polymers, merely their displacement (shear) relative to one another, and would not lead to an overall weakening of wall structure during wall expansion because the disrupted hydrogen bonding can, potentially, re-form in new positions. Also such a mechanism would not involve the release of small sugar fragments. These characteristics are consistent with the known properties of expansins.

In support of this model, we recently found that expansins weaken the mechanical strength of cellulose paper without cellulose hydrolysis (McQueen-Mason and Cosgrove, 1994). Since paper derives its strength from hydrogen bonding between the surface glucans of cellulose microfibrils, it appears that expansins indeed have the ability to disrupt hydrogen bonds between glycans. Our current study shows that pure crystalline cellulose does not bind expansins very well but becomes a strong binding agent after coating with hemicelluloses. Therefore, in the native plant cell wall, we hypothesize that expansins bind to the interface between microfibrils and coating polysaccharides and facilitate wall expansion by catalyzing the disruption of hydrogen bonds between the two, thereby facilitating turgor-driven slippage between microfibrils and other components of the wall.

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