Two Endogenous Proteins That Induce Cell Wall Extension in Plants

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Plant cell enlargement is regulated by wall relaxation and yielding, which is thought to be catalyzed by elusive "wall-loosening" enzymes. By employing a reconstitution approach, we found that a crude protein extract from the cell walls of growing cucumber seedlings possessed the ability to induce the extension of isolated cell walls. This activity was restricted to the growing region of the stem and could induce the extension of isolated cell walls from various dicot stems and the leaves of amaryllidaceous monocots, but was less effective on grass coleoptile walls. Endogenous and reconstituted wall extension activities showed similar sensitivities to pH, metal ions, thiol reducing agents, proteases, and boiling in methanol or water. Sequential HPLC fractionation of the active wall extract revealed two proteins with molecular masses of 29 and 30 kD associated with the activity. Each protein, by itself, could induce wall extension without detectable hydrolytic breakdown of the wall. These proteins appear to mediate "acid growth" responses of isolated walls and may catalyze plant cell wall extension by a novel biochemical mechanism.

INTRODUCTION

For many years, "wall-loosening" enzymes have been implicated in the control of plant cell enlargement (growth), largely on the basis of rapid biophysical and biochemical changes in the wall during auxin-induced growth (reviewed by Taiz, 1984). Plant walls contain numerous hydrolytic enzymes, which have been viewed as catalysts capable of weakening the wall to permit turgor-driven extension (reviewed by Fry, 1988, 1989). In support of this hypothesis, Huber and Nevins (1981) and Inoue and Nevins (1991) found that antibodies raised against wall proteins could inhibit both auxin-induced growth and wall autolysis of corn coleoptiles. In addition, isolated walls from many species extend irreversibly when placed under tension in acid conditions (Rayle et al., 1970; Hager et al., 1971; Cleland et al., 1987), in a manner consistent with an enzyme-mediated process (Cosgrove, 1989). Despite these results and other evidence in favor of wall-loosening enzymes, a crucial prediction of this hypothesis has never been demonstrated, namely that exogenously added enzymes or enzyme mixtures can induce extension of isolated walls. To the contrary, Ruesink (1969) reported that exogenous wall hydrolytic enzymes could mechanically weaken the wall without stimulating extension. Similarly, autolysis of walls during fruit ripening does not lead to cell enlargement. Thus, a major piece of evidence in favor of wall-loosening enzymes as agents of growth control has been lacking.

In this study, we present evidence that the walls of growing cucumber seedlings possess extractable proteins which can induce extension of isolated walls, and we identify two specific wall-associated proteins with this activity.

RESULTS

Wall Proteins Restore Extension Activity to Inactivated Walls

Our basic approach was to solubilize ionically bound proteins from growing cell walls and to assay their ability to restore endogenous extension activity to heat-inactivated walls. Extension was assayed using a constant load extensometer in which wall samples were clamped under constant force and their extension recorded using an electronic displacement transducer (Cosgrove, 1989). Protein was solubilized from wall fragments isolated from the growing hypocotyls of dark-grown cucumber seedlings. After various unsuccessful attempts at reconstitution, we obtained a crude salt-solubilized fraction with the ability to induce the extension of inactivated walls, as shown in Figure 1A. The extension induced by this extract mimicked that of native extension activity in magnitude and kinetics; i.e., initially high rates decayed over a period of 2 hr to more stable rates of 2 to 4% per hr. These rates are lower than elongation rates of the living stem, but the stress applied to the isolated walls was only one-fifth of the equivalent longitudinal stress imposed on the walls by cell turgor (Cosgrove, 1989). Like the endogenous extension activity of isolated cell walls, the reconstituted activity required an acidic pH and was irreversible (i.e.,

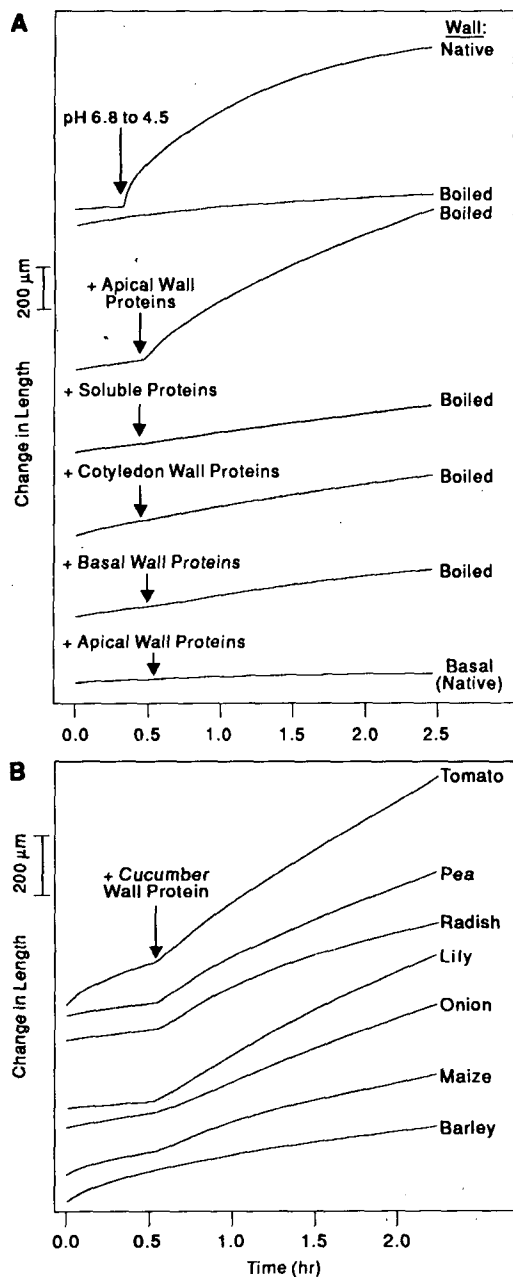[1] To whom correspondence should be addressed.

Figure 1. Extension of Native and Reconstituted Cell Wall Specimens under Constant Load.

Apical 10-mm sections of tissue were frozen, thawed, abraded, and pressed (to remove cell sap and aid in handling) prior to suspension in the extensometer. Samples were clamped under an applied force of 20 g, and extension was recorded using a linear voltage displacement transducer (Cosgrove, 1989). The specimen length between the clamps was 5 mm.

(A) Extension of native and reconstituted cucumber hypocotyl walls. From top to bottom: native walls were suspended under tension in 50 mM Hepes, pH 6.8, for 20 min, after which the bathing solution was replaced by 50 mM sodium acetate, pH 4.5 (arrow). To inactivate the

upon removal of load, the walls did not return to their original length). The addition of protein to native cell walls did not substantially enhance extension, suggesting that endogenous activity was saturating or that binding sites were not accessible to the added material.

Activity Is Associated with Walls of Growing Tissues

The extractable wall extension activity appeared to be specifically associated with the wall. When the soluble cytoplasmic fraction was collected as expressed cell sap or as homogenate from growing hypocotyls, it showed no activity (Figure 1A), whereas proteins that were salt solubilized in an equal volume from washed walls taken from the same tissue showed clear wall extension activity.

The active material appeared to be restricted to the growing region of the hypocotyl. When wall fragments from basal (nongrowing) stem tissue or from the cotyledons (which expand negligibly in our conditions) were extracted, proteins solubilized in the same way did not induce wall extension (Figure 1A). These results suggest that nongrowing tissues lack the active material; however, we cannot exclude the possibility that the active material was present in the tissue but more firmly bound, inactivated during extraction, or lost during wall isolation.

Basal regions of the hypocotyl do not elongate and the walls from this region likewise lack native wall extension activity

walls, specimens were treated for 15 sec with boiling water prior to clamping; as shown in the second line, this treatment eliminated acid-induced extension. For reconstitution experiments, inactivated walls were suspended in 50 mM sodium acetate, pH 4.5, for approximately 30 min, at which point the bathing solution was replaced by 0.5 mL of fresh solution (arrows) containing 2 to 3 mg of proteins extracted from growing cell walls (apical wall proteins), or with soluble proteins from growing cells (soluble proteins), or proteins extracted from cell walls from the nongrowing cotyledon (cotyledon wall proteins) or from walls of the basal hypocotyl (basal wall proteins). The bottom curve shows that extension was not induced in native (not boiled) cell walls from the basal region of the hypocotyl when treated with an active extract from apical cell walls.

(B) Reconstitution of extension activity in inactivated (heat-treated) walls from growing tissues of different species by the active cucumber wall extract. Walls from the growing region from tomato, pea, and radish hypocotyls, lily and onion leaves, and coleoptiles of maize and barley were prepared, heat treated, and clamped as described above, except that a load of only 10 g was applied to the more fragile tomato and radish walls. Walls were first suspended in 50 mM sodium acetate, pH 4.5, for 30 to 50 min; at the time indicated by the arrow, the bathing solution was replaced with 0.5 mL of the same buffer containing 2 to 3 mg of active wall protein from cucumber hypocotyls (apical 3 cm).

In **(A)** and **(B)**, the kinetics of induction of extension upon the addition of proteins is consistent with the time expected for diffusion of proteins into the wall.

(Cosgrove, 1989). The active wall extract from growing cucumber walls did not induce extension of walls from the basal (nonelongating) stem (Figure 1A). Evidently, during maturation the wall is biochemically modified so that it is not susceptible to extension by this material. Perhaps peroxidative cross-linking of lignin or structural proteins such as extensin (Smith et al., 1986; Cassab and Varner, 1988) is involved in this loss of sensitivity.

Cucumber Extract Is Effective with Walls from Other Species

The extension activity showed an interesting pattern of species specificity. Figure 1B shows that the cucumber wall extract was active on the walls of various dicot seedlings (pea, radish, cucumber, and tomato) and on monocots of the Amaryllidaceae (onion and zephyr lily). In contrast, the extract had a much smaller effect on the coleoptile wall of graminaceous monocots (maize and barley). Because graminaceous monocot cell walls differ from those of dicots by having less pectin and hydroxyproline-rich glycoprotein, and also by having a different type of hemicellulose (McNeil et al., 1984; Fry, 1988), it may be that the active fraction extracted from cucumber walls interacts with one or more of these components to induce extension. It may also be that these grass cell walls are cross-linked (not necessarily covalently) in a manner that renders them immune to the cucumber extract. In contrast, monocots of the Amaryllidaceae have a cell wall composition more similar to dicots than to the Gramineae (Redgwell and Selvendran, 1986), and this may explain their susceptibility to the cucumber-derived activity.

Protein Fractionation Yields Two Proteins with Extension-Inducing Activity

The cucumber wall extract was separated by ammonium sulfate precipitation followed by sequential HPLC, as shown in Figure 2, first using a hydrophobic interactions column, where a single peak of activity (designated as the C3 fraction) was obtained, and then using a cation exchange column from which the activity was eluted as two distinct peaks. These fractions were designated S1 and S2 with respect to their order of elution. Figure 3 shows an analysis of the active fractions by SDS-PAGE that revealed a major band with a relative molecular mass of 29 kD associated with S1, while S2 contained a major band at 30 kD. Active extracts have also been separated by native PAGE and by liquid chromatography with hydroxyapatite, gel filtration media, and DEAE anion exchangers, where activity was consistently associated with these two bands (data not shown). The S1 fraction required only 0.3 to 1.0 μg of protein to reconstitute extension rates similar to that of native extension, whereas the S2 fraction required 1.0 to 2.0 μg.

To look for additional proteins that might contribute to wall

extension activity, we looked for synergistic or combinatorial effects of different fractions by combining all C3 fractions together and all sulfopropyl fractions together and assaying for wall extension activity. The wall extension activites of these combined fractions were only those expected from the additive contributions of the active C3, S1, and S2 fractions identified above.
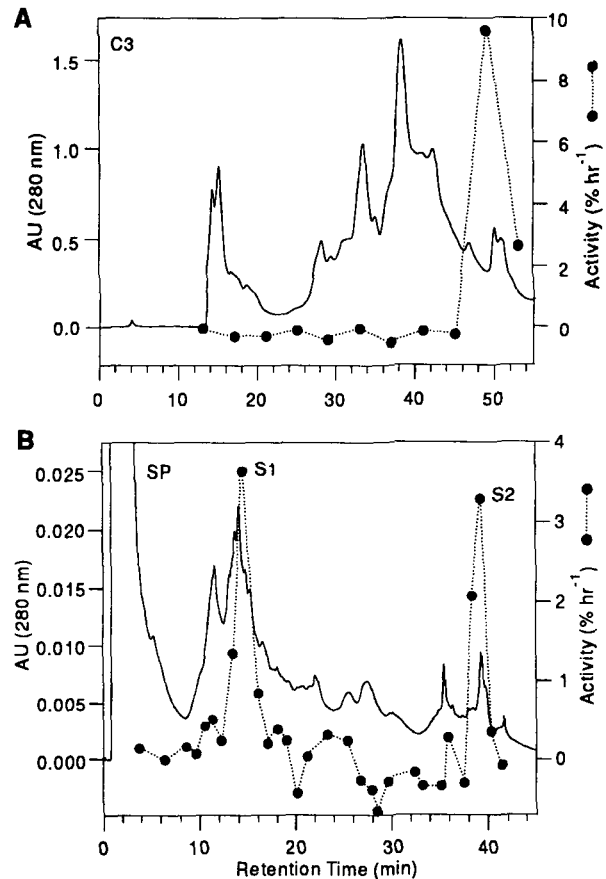

Figure 2. Fractionation of Extension-Inducing Cucumber Wall Extracts by HPLC.

(A) Ammonium sulfate precipitates of salt-extracted cucumber wall extracts were resuspended and loaded onto a C3 hydrophobic interactions column. Proteins were eluted in a descending gradient (0.113 to 0 g/mL) of ammonium sulfate in 50 mM sodium acetate, pH 4.5. Fractions were desalted and checked for extension-inducing activity with inactivated cucumber walls, as described in Figure 1. Activity was calculated as increase in extension rate divided by the length of the specimen (units, % per hr) and is shown as a broken line. Absorbance (AU) at 280 nm is shown by a solid line.

(B) Active fractions from **(A)** were concentrated and desalted into 15 mM Mes, pH 6.5, and loaded onto a sulfopropyl (SP) cation exchange column equilibrated with the same buffer. Proteins were eluted with an ascending gradient of NaCl (0 to 1 M) in this buffer. Extension-inducing activity (broken line, calculated as given in **[A]**) of fractions was measured directly after adjusting the pH to 4.5 with 1 M acetic acid.

Reconstituted Extension Resembles Native Acid-Induced Extension

Endogenous acid-induced extension in cucumber walls was previously shown to be sensitive to a number of exogenously applied factors (Cosgrove, 1989). The thiol-reducing agent DTT stabilized or even enhanced the endogenous extension of isolated cucumber cell walls at acidic pH. Figure 4A shows that DTT similarly enhanced the wall extension activity of cucumber walls reconstituted with an active C3 protein fraction.

Copper and aluminum ions were strongly inhibitory to endogenous acid-induced extension (Cosgrove, 1989), and they likewise inhibited the extension activity reconstituted with C3 proteins (Figure 4A). On the other hand, when inactivated walls were first preincubated in 1 mM Al^{3+} or Cu^{2+} and rinsed briefly to remove unbound ions, active C3 fractions were effective in restoring wall extension activity (data not shown). These results suggest that Al^{3+} and Cu^{2+} exert their inhibitory effect by binding to the active C3 proteins rather than to pectins or other structural components of the wall.

An unusual property of the endogenous wall extension activity is its ability to survive boiling in methanol but not boiling in water (Cosgrove, 1989). Figure 4A shows that methanol-boiled walls retained extractable activity, whereas extracts from walls boiled in water lacked activity. Fractionation of the active extract from methanol-boiled walls by the methods given above showed it to contain the 29- and 30-kD proteins in the S1 and S2 fractions (data not shown). The ability of the activity to survive boiling methanol may be due to the small size of these proteins and to protection within their carbohydrate-rich sites within the cell wall.
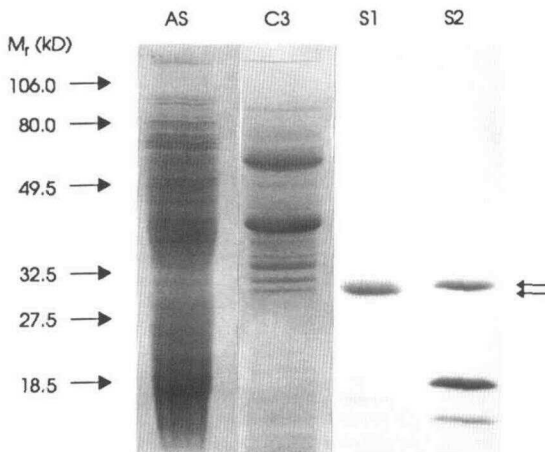

Figure 3. SDS-PAGE of Ammonium Sulfate Precipitate (AS) and Active Fractions from C3 and Sulfopropyl (S1 and S2) HPLC Separations.

Protein samples were concentrated, desalted, and run on SDS-polyacrylamide gels according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R 250. Arrows indicate the major protein bands at 29 and 30 kD in the S1 and S2 fractions that appear to possess the extension-inducing activity.
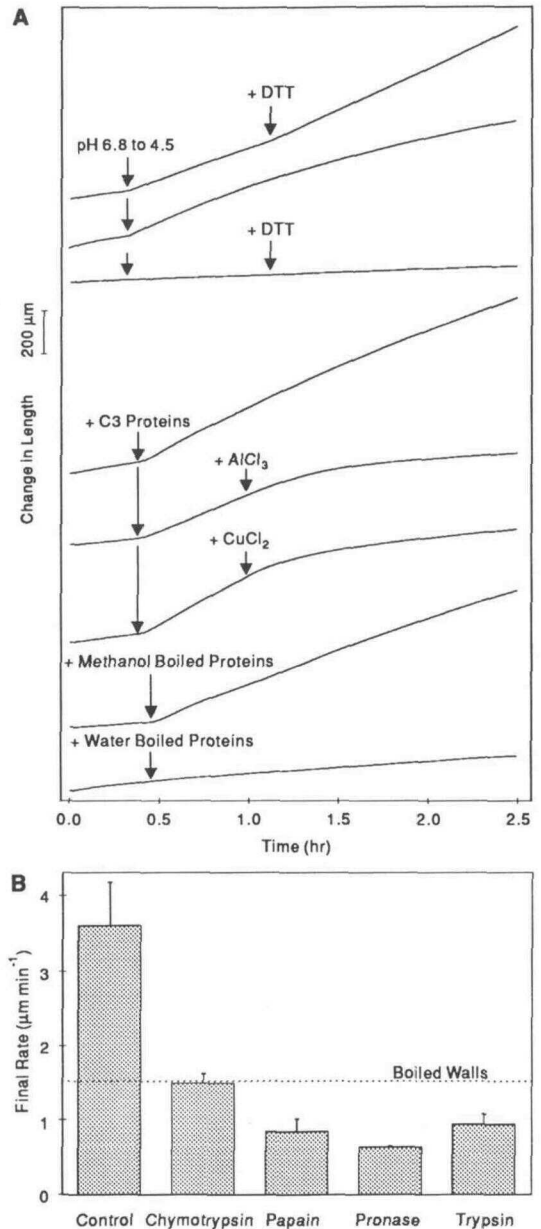

Figure 4. The Effects of DTT, Metal Ions, Methanol and Water Boiling, and Protease Treatments on Reconstituted Extension Activity of Cucumber Walls.

(A) Effects of DTT, metal ions, and heat treatments on reconstituted activity. From top to bottom: to assess DTT effects, cucumber wall specimens were prepared as described in Figure 1A, reconstituted by shaking for 30 min in a solution of active C3 proteins (estimated concentration of 50 µg/mL), and then clamped under constant load in a bathing solution of 50 mM Hepes, pH 6.8. After 20 min, the solution was changed to 50 mM sodium acetate, pH 4.5 (first arrow), and after an additional 40 min, DTT from a 100 mM stock solution was added to give a final concentration of 10 mM. The top two curves represent typical traces from four experiments each with and without added DTT. The third curve shows that DTT by itself has no effect on the extension

Cosgrove (1989) also showed that proteases eliminated endogenous wall extension activity. Figure 4B shows that reconstituted extension activity is similarly inhibited by incubations with chymotrypsin, papain, pronase, and trypsin. When boiled walls were pretreated with these proteases, washed to remove the proteases, and then treated with active C3 proteins, the walls extended at high rates (data not shown). These results indicate that the active components in our wall extracts are proteinaceous and that wall structural proteins (at least those accessible to exogenous proteases) are not necessary for the reconstituted wall extension activity.

To determine the pH sensitivity of the reconstituted activity, experiments were carried out at a range of pH values. Figure 5 shows that there was little or no induction of wall extension by C3 proteins at neutral pH and that extension activity had a maximum between pH 4.5 and 3.5. This pH dependence is similar to that reported for endogenous wall extension activity of cucumber walls (Cosgrove, 1989) and is consistent with the acid growth theory (reviewed by Rayle and Cleland, 1992).

Figure 6 shows that the rate of wall extension increased as the concentration of C3 protein in the surrounding buffer was increased and approached a maximum (saturated) rate which exceeded the rate of native wall extension under comparable conditions by 50 to 100% (Cosgrove, 1989). It was evident that the walls were not binding all of the active proteins because when the protein solutions were transferred at the end of the assay to a new wall specimen, they induced wall extension activity, albeit at a somewhat lower rate.
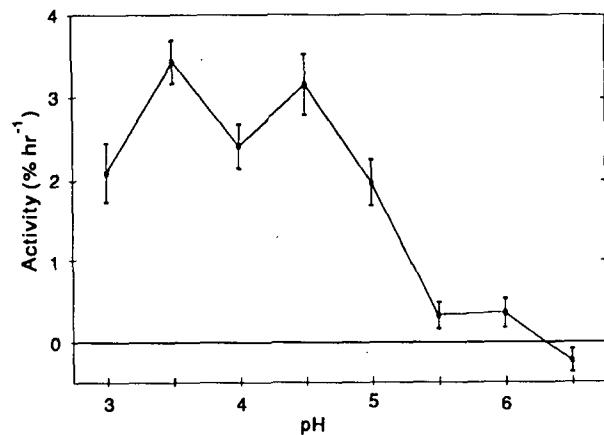

Figure 5. Dependence of Reconstituted Extension on pH.

Cucumber wall specimens, inactivated by heat, were clamped under constant load (as described in Figure 1). Initial bathing solutions were 50 mM citric acid titrated to various pH values with 1 M K_2HPO_4. After 30 min, the bathing solution was changed for a 1:1 dilution of active C3 proteins with the appropriate buffer (final estimated protein concentration was 50 μg/mL); where necessary, the final pH was adjusted using either 1 M citric acid or 1 M K_2HPO_4. Extension was recorded for an additional 2 hr, and activity was calculated as (final rate minus initial rate) divided by wall length.

Figure 4. (continued).

of unreconstituted walls (i.e., boiled but not treated with C3 proteins). To assess the effects of metal ions, wall specimens were inactivated by heat and then clamped under constant load in 50 mM sodium acetate, pH 4.5, as described in Figure 1A. After 20 min, the bathing solution was exchanged for a fresh one containing 50 μg of active C3 proteins per mL (first arrow). After an additional 40 min, $AlCl_3 \cdot 6H_2O$ or $CuCl_2 \cdot 2H_2O$ from 100 mM stock solutions was added to bring the bathing solution to a final concentration of 1 mM (second set of arrows). All experiments were repeated four times. To assess the effects of boiling in methanol or water on the recovery of extension-inducing activity, 100 g of growing cucumber hypocotyl tissue was first boiled for 5 min in 2 L of methanol or for 30 sec in 2 L of distilled water. Tissues were homogenized and wall fragments were recovered and washed. Proteins were salt extracted, precipitated, resuspended, and tested for activity as described in Figure 1. The bottom two lines are representative data from four experiments.

(B) Wall specimens were inactivated by heat and reconstituted with C3 proteins (as described in **[A]**). Reconstituted walls were incubated with 1000 units of trypsin or 2 mg of chymotrypsin for 4 hr at 30°C in 1 mL of 50 mM Hepes, pH 7.3, or with 2 mg of pronase or 2 mg of papain for 4 hr at 30°C in 1 mL of 50 mM sodium acetate, pH 5.0. Controls were reconstituted and incubated in the same manner without the addition of proteases. At the end of the incubations, tissues were clamped under constant load in 50 mM Hepes, pH 6.8. Then, after 30 min, the bathing solution was replaced by 50 mM sodium acetate, pH 4.5. Data presented are the mean ($\pm$SE) extension rates of four experiments in each case.

Extension-Inducing Enzymes Do Not Exhibit Glycanase Activity

Because plant cell walls contain numerous polysaccharide hydrolases hypothesized to weaken the wall and permit turgor-driven extension (Goldberg, 1975; Fry, 1989), we examined the abilities of the highly purified S1 and S2 fractions to release soluble sugars from the wall. The results are shown in Figure 7. During incubation for 4 hr at pH 4.5, native walls released large quantities of monosaccharides and oligosaccharides in a time-dependent manner, as observed in other studies (Goldberg, 1975; Huber and Nevins, 1981). This release was eliminated by a 15-sec incubation in boiling water, a treatment that also eliminated extension activity (Figure 1). The addition of either S1 or S2 fractions to heat-inactivated walls induced rapid extension of wall specimens, but induced no detectable release of monosaccharides or oligosaccharides (Figure 7). Evidently, the active material possessed little or no exo-glycanase activity. Because endoglycanases usually cause the release of small sugar fragments (Maclachlan, 1988) that would be detected in our assay, these results may also be taken as evidence against the involvement of typical endoglycanases. However, these results cannot exclude the possibility that the proteins possess endoglycanase or transglycosylase activity in which short fragments are not released. Long-term (8-hr) incubations of cucumber hemicellulose fractions with either S1 or S2 showed no appreciable shift in molecular mass distribution of hemicelluloses, as assayed by gel permeation
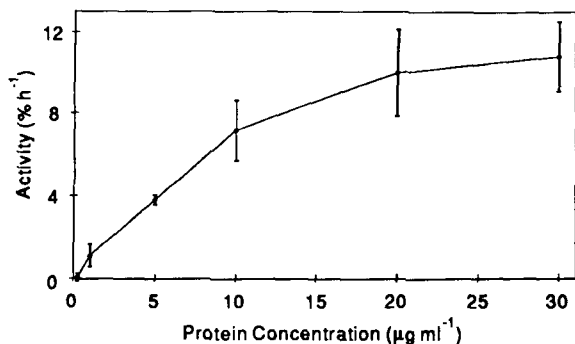
Figure 6. Wall Extension Activity as a Function of C3 Protein Concentration.

Cucumber walls were heat inactivated and reconstituted with 0.5 mL of 50 mM sodium acetate buffer, pH 4.5, containing various concentrations of active C3 fraction proteins. Extension rates were measured before the addition of proteins and 2 hr after protein addition. Activity is expressed as the difference in these rates divided by the length of the segment.

chromatography (data not shown). An endoglycolytic mode of action seems therefore unlikely, but further tests will be needed to confirm this tentative conclusion.

DISCUSSION

We have identified two proteins that may function as the hypothetical wall-loosening enzymes for plant cell growth. Our results showed that cucumber hypocotyl walls possess extractable proteins that can induce extension in heat-inactivated cell walls. The extractable activity is restricted to the walls of rapidly growing cells and can induce extension in walls from other plant species, but is ineffective against mature cell walls. These results are consistent with a role in endogenous plant cell growth. Upon sequential fractionation of the crude extract, two proteins with a similar molecular mass (29 and 30 kD) were associated with the extension activity. Each of these proteins, by itself, could induce extension of isolated walls. They thus possess a crucial property of the long sought after wall-loosening enzymes.

Although wall hydrolases and related enzymes have often been called wall-loosening enzymes (Fry, 1989; Nishitani and Tominaga, 1992), they have never been shown to induce long-term extension of isolated walls. We could detect no polysaccharide hydrolytic activity associated with our purified proteins, which suggests that they may induce extension by some novel biochemical mechanism. Further work is needed to assess their molecular mode of action.

An important question is the significance of this activity, observed with isolated cell walls at acidic pH, to the normal enlargement of plant cells. Earlier work (Taiz, 1984; Cleland et al., 1987; Cosgrove, 1989) showed that this type of in vitro

wall extension resembled in vivo cell enlargement in several respects and was correlated with growth under various conditions. Although the acid growth hypothesis for auxin action remains controversial (Schopfer, 1989; Luthen et al., 1990; Rayle and Cleland, 1992), there is little argument that cell walls have a pH in the range of 4 to 6, where the activity of these proteins showed a strong pH dependence, and that wall acidification via external buffers or via fusicoccin stimulates faster
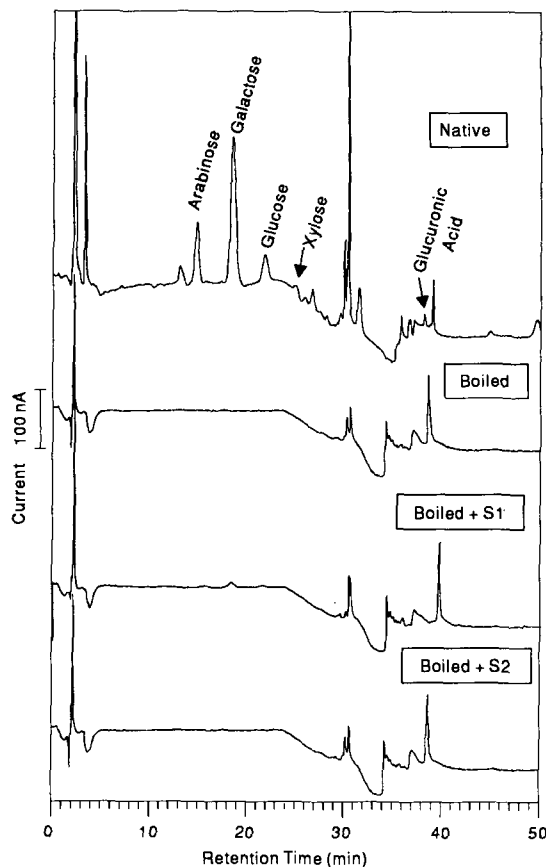

Figure 7. Autolytic Sugar Release by Native, Boiled, and Reconstituted Cucumber Wall Specimens.

Cucumber wall specimens were prepared as described for Figure 1, thoroughly washed to remove soluble sugars, and incubated in 50 mM sodium acetate, pH 4.5, at 30°C for 4 hr. Aliquots from the incubation were separated on an anion exchange column and quantified using a pulsed amperometric electrochemical detector. Inactivated walls were boiled for 15 sec in water and subsequently handled in the same way, or incubated with quantities of S1 or S2, which were shown to give high rates of extension in parallel reconstitution assays. The peaks shown for the inactivated walls appeared to be due either to trace buffer contaminants or artifacts of the steep gradient. No reproducible sugar release from the walls could be detected upon addition of the S1 or S2 fractions to the walls. The miniscule peak appearing at 19 min in the "Boiled + S1" run proved to be found in the S1 fraction itself and was not a time-dependent release from the walls.

growth (Kutschera and Schopfer, 1985; Ridge and Osborne, 1989; Hager et al., 1991). Because of the similar sensitivity of native and reconstituted wall extension activities to pH, DTT, proteases, methanol boiling, and metal ions, we concluded that the proteins identified here mediate the long-term acid growth response of isolated cucumber walls.

Hager et al. (1991) have recently proposed a variant of the acid growth hypothesis, in which auxin stimulates cell enlargement by increasing the amount of plasma membrane H^+-ATPase, which in turn acidifies the wall space and thereby loosens the wall. The proteins we have identified seem likely to mediate the wall loosening in this scenario, although our data only indirectly support this idea. Because auxin also increases the capacity of isolated walls to extend at acidic pH (Cleland, 1983), auxin's long-term action may involve increased synthesis of these wall proteins. Auxin rapidly induces the synthesis of a number of mRNA species of unknown function (Guilfoyle, 1986; Theologis, 1986), some of which may correspond to the proteins found here (McClure et al., 1989).

How do these proteins induce cell wall extension? Three observations suggest they act catalytically rather than as structural material. First, the endogenous extension activity of isolated cucumber cell walls—which appears to result from the activity of these proteins—persisted for many hours without synthesis or the addition of new materials (Cleland et al., 1987; Cosgrove, 1989) and resulted in extension of more than 40% (at which point breakage then occurred at the site where the holding clamp bit the sample). Second, the concentration of exogenous protein required to restore native extension rates was as low as 0.3 μg (10^{-8} mol, assuming a molecular mass of 30 kD). Third, after the exogenous proteins induced wall extension, the external solution could be exchanged for one without protein and the extension continued, indicating that the added protein was bound to the walls and exerted its action without the further addition of protein.

The biochemical mechanism of action of these proteins is of keen interest because the process of plant cell wall extension is not yet understood in terms of structural rearrangements of the wall polymers. Our results indicate that these proteins are not acting as exoglycanases or as typical endoglycanases. By analogy with the "hatching enzymes" of Chlamydomonas (Matsuda, 1988) and the autolysins of bacterial walls, they might act as peptidases to break the covalently bound web of structural proteins believed to surround plant cells (Smith et al., 1986; Cassab and Varner, 1988; Fry, 1989). Alternatively, they might act without breaking covalent bonds by binding specific wall components, thereby interfering with noncovalent binding between wall polymers and allowing extension in response to wall stress. Further analysis of these proteins is in progress.

These appear to be novel wall proteins with the ability to induce extension of isolated plant cell walls. Biophysical studies indicate that plant cell enlargement is controlled by a dynamic and complex process, with perhaps more than one control point (Taiz, 1984; Cosgrove, 1987). We suggest that these two proteins may play an essential role in the control of wall extension in vivo, namely as accelerators of wall extension. If so,

then their activity may be modulated by endogenous growth hormones, light, gravity, and other stimuli that alter cell enlargement rates.

METHODS

Plant Materials

Seeds of cucumber (Cucumis sativus cv Burpee Pickler) were sown on Kimpak Seed Germination Paper K-22 (Seedburo Equipment Company, Chicago, IL) soaked with distilled water, in flats, 50 × 25 × 6 cm, with lids of the same dimensions. Seedlings were grown in the dark for 4 days at 27°C. The apical 3 cm of hypocotyl was excised and frozen at −20°C for no more than 5 days and prepared for extension measurements as previously described (Cosgrove, 1989). Basal walls were from the lower 6 cm of the (15 cm long) hypocotyls.

Seeds of pea (Pisum sativum cv Alaska), radish (Raphanus sativus cv Crimson Giant), tomato (Lycopersicon esculentum cv Rutgers), onion (Allium cepa cv Snow White), maize (Zea mays cv B73 × Mo17), and barley (Hordeum sativum cv Barsoy) were sown on vermiculite wetted with distilled water and grown for 4 days in the dark at 27°C. Hypocotyls of pea, radish, and tomato; primary leaves of onion; and coleoptiles of maize and barley were excised and frozen for subsequent extension experiments. Young growing leaves of zephyr lily (Zephyranthes candida) were removed from a greenhouse-grown colony of plants, which were kept in the dark at 27°C for 12 hr prior to harvest, and frozen for later extension assay.

Extension Measurements

Wall extension was measured with a constant load extensometer as described by Cosgrove (1989). Briefly, frozen/thawed tissues were abraded with carborundum (320 grit, washed well before use; Fisher Scientific, Fair Lawn, NJ) to disrupt the cuticle, boiled in water for 15 sec (for reconstitution assays), and secured between two clamps (with about 5 mm between the clamps) under a constant tension of 20 g (for assays with radish, tomato, and onion tissues, the force was reduced to 10 g). Plastic cuvettes were fitted around the walls and filled with 0.5 mL of bathing solution. For reconstitution assays the bathing solution was first 50 mM sodium acetate, pH 4.5, for 30 min, followed by the protein fraction to be assayed in a buffer at the same pH. Movement of the lower clamp was detected with an electronic position transducer and recorded on a microcomputer. All extension assays reported here were repeated at least five times, except for the heterologous reconstitutions (e.g., with walls from pea, tomato, and corn), which were performed three to five times.

Protein Extraction and Fractionation

For bulk wall extractions, the apical 3-cm hypocotyl regions of cucumber seedlings (approximately 150 to 200 g of tissue) were collected on ice water and homogenized with 20 mM sodium acetate, 2 mM EDTA, pH 4.5, in a Waring blender. The wall fragments were collected by filtration through Miracloth or a nylon screen (70-μm mesh), washed twice with buffer, and extracted for 1 to 24 hr (typically overnight) in 20 mM Hepes, pH 6.8, 1 M NaCl, 2 mM EDTA, 3 mM sodium metabisulfite at 4°C. Cell wall fragments were removed by filtration, and the salt-

solubilized fraction was precipitated with ammonium sulfate (the activity precipitated between 0.113 and 0.390 g/mL of $[NH_4]_2SO_4$). The resuspended precipitate was desalted on a 7-mL column of Bio-Gel P-2 (Bio-Rad) into 50 mM sodium acetate, pH 4.5. Protein concentration was 2 to 4 mg/mL, estimated by Coomassie Protein Assay Reagent (Pierce, Rockford, IL).

For the comparison of soluble and wall-associated proteins (Figure 1A), 100 g of tissue was harvested and homogenized with 100 mL of 20 mM sodium acetate, pH 4.5, 1 mM EDTA. Wall fragments were filtered out, and the remaining solution was designated as the soluble fraction. Wall fragments were washed with buffer and then extracted in 200 mL of 20 mM Hepes, pH 6.8, 1 M NaCl, 2 mM EDTA, 3 mM sodium metabisulfite for 1 hr at 4°C. Aliquots of both solutions were dialyzed against 50 mM sodium acetate, pH 4.5, and tested for wall extension activity.

For HPLC fractionation, protein in the wall extract was precipitated with 0.390 g/mL $(NH_4)_2SO_4$ and resuspended to a volume of 2 mL with 50 mM sodium acetate, pH 4.5, 0.084 g/mL $(NH_4)_2SO_4$. Insoluble material was removed by centrifugation and by filtration through an Acrodisc Supor PF syringe filter (Gelman Sciences, Ann Arbor, MI) prior to being loaded onto a C3 hydrophobic interactions column (ISCO C-3/6.5 μm 10 × 250 mm) equilibrated with 50 mM sodium acetate, pH 4.5, 0.113 g/mL $(NH_4)_2SO_4$. Proteins were eluted from the column with a linear gradient from the equilibration buffer into 50 mM sodium acetate, pH 4.5, in 35 min at a flow rate of 1 mL/min. Fractions were desalted on a 7-mL column of Bio-Gel P-2 into 50 mM sodium acetate, pH 4.5, and assayed for wall extension activity.

The active fractions from the C3 column were pooled and subsequently desalted and concentrated on a Centricon-30 microconcentrator (Amicon, Beverly, MA) or an Ultrafree-20 10,000 nominal molecular weight limit (Millipore, Bedford, MA) centrifugal filter unit, the buffer being exchanged for 15 mM Mes NaOH, pH 6.5. The concentrated sample was then loaded (in a volume of 1.7 mL) onto a sulfopropyl cation exchange column (Bio-Rad HRLC MA7S 50 × 7.8 mm) equilibrated with 15 mM Mes NaOH, pH 6.5, and proteins were eluted with an ascending gradient of NaCl (from 0 to 1.0 M over 45 min) in the same buffer at a flow rate of 1 mL/min. Absorbance at 280 nm was measured using a Dionex Variable Wavelength Detector (VDM-2) (Dionex Corporation, Sunnyvale, CA).

Proteins from various stages of purifications were concentrated and desalted on Centricon 30 microconcentrators and then run on SDS–polyacrylamide gels according to the method of Laemmli (1970). Gels were subsequently stained with Coomassie Brilliant Blue R 250. Protein analysis by this procedure was repeated more than 10 times with similar results.

For Figure 6, proteins in the active C3 fractions were first precipitated by adding acetone (−20°C) to 67% (v/v), pelleted by centrifugation, and resuspended in 50 mM sodium acetate buffer, pH 4.5. Protein concentrations were assayed by the Coomassie Protein Assay Reagent.

Autolytic Sugar Release

Wall specimens were prepared as described by Cosgrove (1989) and washed thoroughly three times in autoclaved deionized water to remove soluble sugars. Ten segments were incubated for 4 hr in 0.3 mL of 50 mM sodium acetate, pH 4.5, 1 mM sodium azide at 30°C. The incubation solution was subsequently cleared of large molecular mass contaminants by centrifugation through a filter (Millipore Ultrafree-MC, 10,000 nominal molecular weight limit) prior to loading onto the HPLC column. Active S1 and S2 fractions were added to the incubation

medium at an estimated 3 μg/mL. Parallel experiments showed high extension-inducing activity in these fractions.

Sugars were separated on a Dionex AS6 anion exchange column at a flow rate of 1 mL/min and quantified using a Dionex pulsed amperometric electrochemical detector with a 1:1 postcolumn addition of 500 mM NaOH. The elution protocol was as follows: 0 to 20-min elution with 1 mM acetic acid, 2 mM NaOH, followed by an 8-min gradient to 1 mM acetic acid, 100 mM NaOH, followed by a 1-min gradient to 150 mM acetic acid, 100 mM NaOH, and finally 21-min elution with the last eluant.

Sugars were identified by their retention times and by "spiking" of the samples with specific sugars to test for coelution. These assays were performed more than three times with similar results.

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