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Evidence Against the Involvement of Ionically Bound Cell Wall Proteins in Pea Epicotyl Growth[1]

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ABSTRACT

Ionically bound cell wall proteins were extracted from 7 day old etiolated pea (*Pisum sativum* L. cv Alaska) epicotyls with 3 molar LiCl. Polyclonal antiserum was raised in rabbits against the cell wall proteins. Growth assays showed that treatment of growing region segments (5–7 millimeters) of peas with either dialyzed serum, serum globulin fraction, affinity purified immunoglobulin, or papain-cleaved antibody fragments had no effect on growth. Immunofluorescence microscopy confirmed antibody binding to cell walls and penetration of the antibodies into the tissues. Western blot analysis, immunoassay results, and affinity chromatography utilizing Sepharose-bound antibodies confirmed recognition of the protein preparation by the antibodies. Experiments employing *in vitro* extension as a screening measure indicated no effect upon extension by antibodies, by 50 millimolar LiCl perfusion of the apoplast or by 3 molar LiCl extraction. Addition of cell wall protein to protease pretreated segments did not restore extension nor did addition of cell wall protein to untreated segments increase extension. It is concluded that, although evidence suggests that protein is responsible for the process of extension, the class(es) of proteins which are extracted from pea cell walls with 3 molar LiCl are probably not involved in this process.

Expansive growth of plant cells requires a weakening or loosening of the wall, yielding of the wall, and uptake of water. The biochemical basis for wall loosening remains uncertain, although it appears to require active metabolism and protein synthesis (for review, see Taiz [32]). Indirect evidence supports the notion that wall enzymes break one or more load-bearing links (8), but identification of such wall-loosening enzymes has been elusive. Autolytic enzymes have been found in the wall (15–17, 23, 31), and in some cases there is a correlation between autolytic activity and growth rate (13, 23), but there has not yet been a direct demonstration that the activity of such enzymes leads to cell growth. For example, Ruesink (31) reported that various polysaccharidases mechanically weakened the oat coleoptile wall, as measured by stress/strain analysis, but did not enhance growth. Thus, the evidence for growth control by wall loosening enzymes remains circumstantial.

In 1981, Huber and Nevins (16) reported that corn coleoptiles treated with antibodies raised against cell wall proteins extracted with 3 M LiCl showed a 35% suppression of growth. This finding suggested a new method of utilizing antibodies as a tool to determine which protein(s)/enzymes(s) were responsible for wall loosening and cell expansion. They also reported a 70% reduction in the autolytic reactions of isolated cell walls; however, antibody treatments did not directly inhibit enzyme activities. They suggested that antibodies impaired the mobility within the wall of autolytic enzymes necessary for wall loosening. Further investigation has led Nevins *et al.* (27) to propose that some proteins involved in growth are not autolytic. Antibodies raised against a protein which does not possess endo- or exoglucanase activity, and which has yet to be shown to possess any kind of enzymic activity, can suppress growth in corn coleoptiles. They suggest that the protein may be a functional lectin and that antibodies may serve to aggregate the protein(s).

Recently, Morrow and Jones (25) showed that antibodies raised against cell wall proteins which were centifuged from pea segments infiltrated with 50 mM $CaCl_2$ had no effect on growth. They were able, by means of immunofluorescence microscopy, to show penetration of the antibodies into the tissue and binding of the antibodies to the cell walls. Additionally, recognition of the protein preparation by the antibodies was shown with Ouchterlony immunodiffusion and Western blot analysis. They concluded that the growth associated protein(s) are tightly bound to the wall and not extractable with 50 mM $CaCl_2$.

In light of these divergent results, we attempted to determine whether antibodies against tightly bound proteins (3 M LiCl extractable) would inhibit the growth of pea epicotyl growing region segments. The design of the investigation was to measure quantitatively the effects of antibodies on growth and *in vitro* extension. Further investigations of the immunoreactivity of the CWP[3] and penetration of the pea tissues by the antibodies were also carried out with both quantitative and qualitative methods.

MATERIALS AND METHODS

Preparation of Protein. Cell wall protein was extracted from plant material following the methods outlined by Huber and Nevins (16). Pea seedlings (*Pisum sativum* L. cv Alaska) (W. Atlee Burpee, Co.) were grown in darkness for 7 d in saturated vermiculite. Approximately 450 g of epicotyl tissue was harvested into 50 mM NaCl at 0°C. The tissue was homogenized in a Waring blender for 3 min, filtered through premoistened Miracloth (Calbiochem) and washed with 2 L 50 mM NaCl at 0°C. The residue was additionally washed with 2 L acetone at −20°C, then with 1 L 50 mM NaCl at 0°C, and suspended in 100 ml 3 M LiCl at 4°C for 48 h. The protein-containing solution was vacuum filtered

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[3] Abbreviations: CWP, cell wall protein; IgG, immunoglobulin G; $F(a,b)_2$ fragments, 50 kD immunoglobulin fragments produced by papain cleavage of IgG; FITC, fluorescein isothiocyanate; Ag-Ab, antigen-antibody complex; HRP, horseradish peroxidase; LVDT, linear variable differential transformer; ANOVA, analysis of variance; RGR, relative growth rate.

from the cell wall material through premoistened Miracloth and dialyzed three times against 4 L 20 mM Na-K phosphate (pH 7.2) + 200 mM NaCl at 4°C for a total of 24 h. The dialyzed protein was stored in aliquots at −20°C. In some cases, a final dialysis against distilled water was carried out and the protein solution lyophilized and stored at −20°C. Average protein yield was approximately 0.6 mg protein/g epicotyl tissue. Protein concentrations were determined using the Peterson micro-Lowry procedure (28) with BSA as the protein standard. Although the wall extract is referred to as CWP, we realize that it is likely to contain large polysaccharide fragments which are probably antigenic.

Immunization of Rabbits. Three female New Zealand White rabbits were immunized with wall protein prepared as described above. Immunization procedures were carried out according to the methods of Vaitukaitis (34). Each rabbit was bled from the marginal ear vein prior to the initial injection to obtain preimmune serum. Approximately 350 μg protein was injected intradermally into each rabbit. Two subsequent booster injections were made at 2 week intervals (397 and 265 μg protein per rabbit, respectively). Sixteen weeks after the first immunization, each rabbit was injected intradermally with 2 mg protein in saline and exsanguinated 3 d later. Serum was recovered as previously described (16).

Growth Assays. Growth assays were carried out using growing region segments from 7 d old etiolated pea seedlings. Growing region segments (5–7 mm) were abraded with a carborundum/water slurry and pretreated in either buffer, preimmune or immune preparations for 90 min. Following the pretreatments, the segments were mounted onto microscope slides and photographed to establish initial lengths. The segments were incubated in 80 × 25 mm glass vials containing 10 ml of 1 mM Mes-NaOH (pH 6.0) ± 5 × 10^{-6} M IAA (Calbiochem) and rotated on a clinostat (10 rpm) to provide gentle aeration. At least 10 segments were used for each of the 6 treatments. Segments were photographed at 1 h intervals for 5 h. Segment lengths were determined with the use of a digitizing tablet connected to a microcomputer (10).

Serum and Antibody Purifications. Dilutions (1:4) of whole serum were dialyzed against 1 mM Na Citrate (pH 6.0) overnight at 4°C. Serum globulin fractions were prepared via ammonium sulfate precipitation (16). Affinity purification of IgG was achieved through column chromatography using Affi-Gel Protein A agarose (Bio-Rad) (2). Columns were washed between separations with 1.5 M Na thiocyanate to prevent reduction of binding capacity. Average IgG recovery was 10.86 mg IgG/ml serum.

Production of $F(a,b)_2$ fragments was carried out by treating a 1 ml sample of 5 mg/ml purified IgG with 30 μl of 0.0068 mg/ml papain (2 × crystallized, Sigma) for 30 min at 25°C (30). Separation of the antibody fragments from the papain was achieved by gel filtration with Bio-Gel P-100 (Bio-Rad). The sample was placed on a 1.5 × 30 cm precalibrated column and run with 0.02 M K-phosphate (pH 7.0). The elution profile (A_{280}) showed a single peak at 50 kD. These fractions were pooled and stored at −20°C. No attempt was made to remove Fc fragments from the preparation.

Immunofluorescence Microscopy. Indirect immunofluorescence procedures were carried out following the methods of Morrow and Jones (25) with FITC-labeled goat anti-rabbit IgG (Sigma). Reduction of nonspecific protein binding was achieved through pretreatment of tissue with 0.5% goat serum (Sigma). Observations and photographs were made using a Nikon Optiphot epifluorescence microscope providing excitation wavelengths of 330 to 380 nm.

Immunoassay Procedures. Iron labeling of cell wall protein was carried out according to the methods of Cais (4) with ferrocenecarboxylic acid (Aldrich) (138 mg) substituted in place of the cobalt carboxylic acid. Atomic absorption indicated a labeling efficiency of 0.026 mg iron/mg protein. A double-antibody immunoassay procedure (24) employing ammonium sulfate precipitation of the Ag-Ab (7) was used to determine the percent recognition of the protein mixture by the purified IgG preparations. Precipitates were digested with 3 ml concentrated H_2SO_4 and diluted to a final volume of 15 ml. Iron was quantified by absorption at 248.8 nm with a Buck 200 atomic absorption spectrophotometer.

Affinity Chromatography. Purified IgG was bound to cyanogen bromide activated Sepharose 6MB (Sigma) as previously described (1). Binding capacity of the conjugated beads was 1.6 mg CWP/0.5 g beads. Antibody-bound beads (0.5 g) were rotated with 1 mg CWP overnight at 4°C in 0.01 M Na-K phosphate (pH 7.2). The supernatant of a low speed centrifugation (50 *g*) and a 10 ml wash of the column (1.4 ml bed volume) were pooled as the void fraction. Bound protein was eluted from the column with 5 ml 0.05 M glycine-HCl (pH 2.8). Void and bound fractions were dialyzed against distilled water overnight at 4°C, lyophilized and resuspended in a minimal volume of 0.1 M Hepes-KOH (pH 7.0). Samples of bound and void fractions were subjected to SDS-PAGE.

Protein Electrophoresis and Western Blots. Proteins were separated on polyacrylamide slab gels according to the methods of Laemmli (21) using a 3.5% acrylamide stacking gel and a 10% acrylamide separating gel. The sample buffer contained 1% 2-mercaptoethanol. Electrophoresis was carried out overnight at a constant amperage of 5 mamp/gel.

Electroblotting of protein onto nitrocellulose (Schleicher and Schuell) was carried out following the procedures of Burnette (3) at a constant amperage of 2.2 amp for 4 h. The blots were stained according to the methods of Hawkes *et al.* (14) substituting 3% casein in place of the BSA used in the blocking procedure. A 1:250 dilution of primary antiserum and a 1:500 dilution of horseradish peroxidase labeled secondary antibody (HRP-goat anti-rabbit IgG obtained from Sigma) was used in localization. The blot was developed with 4-chloro-1-naphthol and H_2O_2.

Apoplast Perfusion. Perfusion of the apoplast of living tissue was accomplished using the pressure perfusion chamber of Cosgrove and Cleland (11). Etiolated pea epicotyl segments (2–3 cm) which included the growing region were secured with epoxy and perfused with 50 mM salt solutions at 10 psi until 125 μl/segment perfused solution was collected. This volume is at least 10 times the apoplast volume of the segment. Following perfusion, the segments were frozen at −20°C in preparation for *in vitro* extension.

In Vitro Extension ('Creep') Assays. Pea epicotyls were cut and frozen at −20°C. The upper portion of the epicotyl was abraded with a carborundum/water slurry while still frozen and 1 cm apical sections were cut after the epicotyls thawed. Pretreatments with various agents were carried out at this point. Excised segments were pressed under a 500 g weight for 10 min to flatten the tissue. Individual segments were clamped into a constant stress extension apparatus (9) with 3 to 5 mm of tissue between the clamps. The movable clamp of the apparatus was attached to an LVDT core and the entire clamp assembly was weighted to provide a 20 g load. The segment was enclosed in a chamber and incubated with 50 mM Hepes-KOH (pH 6.8) for 45 min. The solution was changed to 50 mM Na acetate (pH 4.5) and incubated for 135 min. The resulting extension was recorded and stored on diskettes by a North Star Horizon microcomputer (9). The percent per hour extension was calculated from the slope of the linear phase divided by the initial clamped tissue length.

Pretreatment of frozen/thawed tissue with protease with carried out using 1 mg/ml nonspecific protease from *Streptomyces griseus* (Sigma, Type XIV, P-5147) in 50 mM K-Phosphate (pH 7.5) at 25°C. Protease digestion proceeded for 3 h after which

the tissue was washed three times in 100 ml distilled water for a total of 1.5 h. Boiling pretreatment consisted of plunging frozen/thawed epicotyls into boiling water for 15 s. Two hour pretreatments with antibodies (IgG and $F(a,b)_2$) were made at a concentration of 0.5 mg/ml and cell wall protein pretreatments were made at 1 mg/ml. High salt extraction of frozen/thawed segments was carried out by constant shaking of segments in 3 M LiCl for 3 h. Segments were washed after extraction three times with 100 ml distilled water for a total of 1.5 h.

RESULTS

Growth Effects of Antibodies. Figure 1 summarizes the data from three growth assays conducted with dialyzed serum. Treatment of segments with dialyzed immune serum did not suppress IAA-induced growth. On the contrary, immune pretreated segments + IAA treatment consistently showed a growth rate higher than that of the controls. However, these growth rates were found not to be statistically different from the controls (ANOVA, regression analysis).

In an attempt to eliminate the possible effects of serum components other than antibodies, various purifications of the sera were conducted. Pretreatment of segments with serum globulin fraction (1 mg/ml), purified IgG (1 mg/ml), or $F(a,b)_2$ fragments (1 mg/ml) resulted in growth profiles similar to those seen in Figure 1. Because of their smaller size (50 kD), we assumed $F(a,b)_2$ fragments could penetrate the tissue more easily than whole IgG. Comparisons of relative growth rates from a total of eight growth assays with various pretreatments are made in Figure 2. One way ANOVA and range tests using the Scheffe method of homogenous group determination show no difference between the pretreatments at 95% confidence. The overall growth rates for IAA-induced growth shown in Figure 2 are much lower than that measured for pea segments in which no pretreatments were carried out (data not shown). However, the growth rates are comparable to those measured by Morrow and Jones (90 min pretreatment) (25) and higher than those reported by Nevins *et al.* (2 h pretreatment) (27). It is possible that the abrasion or the 90 min preincubation before auxin treatment is responsible for low growth rates.

Antibody Penetration. Lack of antibody effect on growth might be caused by poor penetration into the tissue. This possibility was investigated by means of immunofluorescence microscopy. In Figures 3 a,a′ and 3 b,b′, pea segments were abraded and treated for 90 min in the same manner as for growth assays. Following the pretreatment, the segments were washed, sectioned, and stained with FITC-goat anti-rabbit IgG. Figure 3a′ shows the low fluorescence of tissue treated with preimmune serum. Arrows indicate areas of autofluorescence in the cuticle and vascular bundles. Very low fluorescence is also visible in the cell walls. Penetration of the antibodies into the tissue is confirmed in Figure 3b′. Uneven staining of the tissue is possibly due to uneven abrasion of the cuticle or due to movement of antibodies through the vascular tissue. Staining of the vascular tissue was a consistent feature of this treatment. Figure 3c′ shows the fluorescence obtained when the antiserum was applied directly to the cut surface of the section. Fluorescence is most prominent in the epidermal layers. The vascular tissue is also stained. In all tissue treatments nonspecific binding was reduced through the application of 0.5% goat serum to the sections before the application of FITC-labeled antibodies and through extensive washings after all protein treatments.

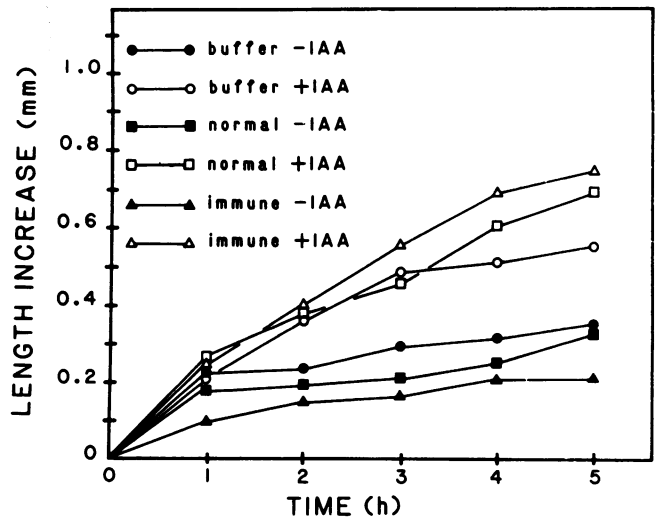

FIG. 1. Data from growth assays conducted following 90 min pretreatment with dialyzed serum. Initial segment length = 7 mm. [IAA], 5×10^{-6} M; buffer, 1 mM Mes-NaOH (pH 6.0). Each plotted point represents the average of 30 segment lengths. Standard errors ranged between 0.03 and 0.07.

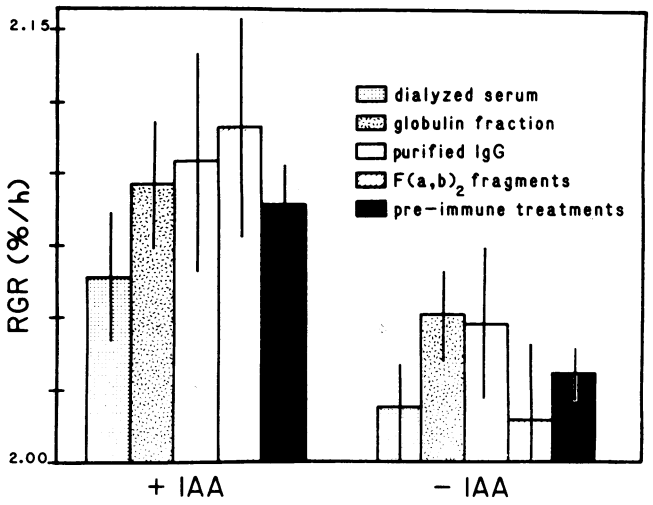

FIG. 2. Relative growth rates of pea segments (±95% confidence intervals). Growth rates calculated following 90 min pretreatments and eliminating expansion values from the first hour of growth (*i.e.*, 2.5–6.5 h after excision). Dialyzed serum, $n = 30$; globulin fraction, $n = 30$; IgG, $n = 10$; $F(a,b)_2$, $n = 10$; preimmune, $n = 80$.

Recognition of CWP by Antibodies. Although antibodies were able to penetrate the tissue, it was possible that most of the CWP was not antigenic. This possibility was tested by several means. Antibody recognition of CWP was examined by immunoprecipitation. Cell wall protein was labeled with ferrocene (dicyclopentadienyl iron) and iron levels were measured by atomic absorption spectrophotometry. The level of iron labeling was 2.60% (w/w) and corresponded to a lower detection limit of 20 μg/ml protein. Immunoassays were conducted using 1 ml of 3 mg/ml CWP + 1 ml of 3 mg/ml purified IgG. Second antibody treatment was made with 1 ml of 3 mg/ml goat anti-rabbit IgG (24). Three ml of 4 M $(NH_4)_2SO_4$ (pH 7.5) was added to enhance the precipitation of the Ag-Ab complex (7). Comparisons of bound to total protein gave a value of 75% precipitable protein. Preimmune IgG resulted in 36% precipitation, indicating the possibility of some nonspecific binding.

Immunoreactivity of the antibodies was also examined qualitatively via affinity chromatography and SDS-PAGE. Purified IgG was immobilized on Sepharose beads, CWP was allowed to react with the Sepharose-Ab and then eluted. The electropho-

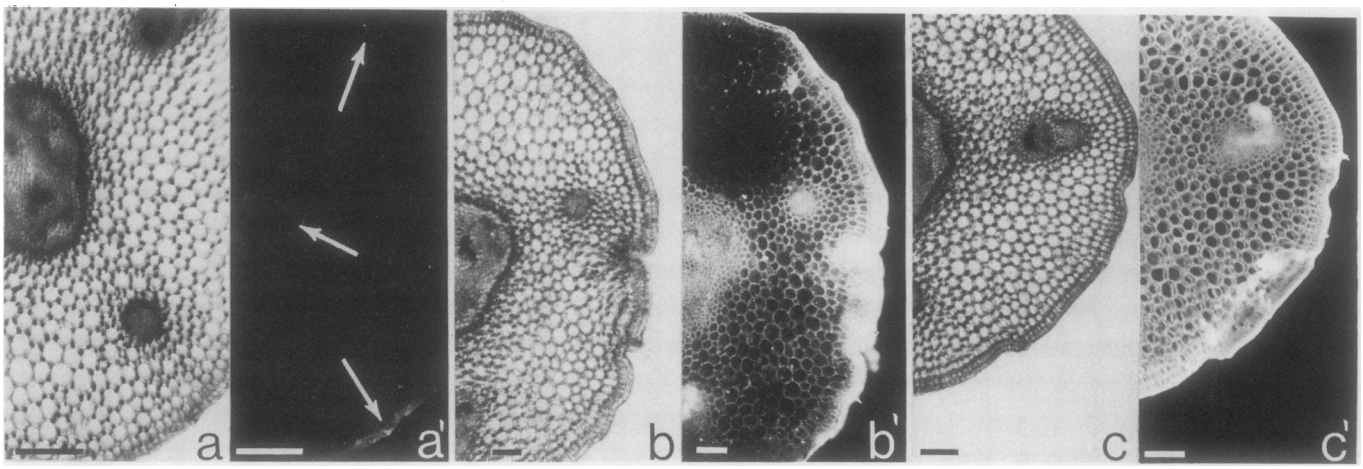

FIG. 3. Immunofluorescence micrographs of pea epicotyl cross sections, a,a' section from an abraded segment incubated in a 1:4 dilution of preimmune serum 90 min, bright field, and fluorescence, respectively (×65). Arrows indicate areas of autofluorescence. b,b' section from an abraded segment incubated in a 1:4 dilution of immune serum 90 min, bright field, and fluorescence, respectively (×65). c,c' 1:4 dilution of immune serum applied directly to the cut surface of tissue, bright field, and fluorescence, respectively (×65). Magnification bars = 25 μm.

retic profiles of the bound and void fractions were compared. The void fraction pattern is seen in Figure 4B and the bound fraction pattern in Figure 4C. The bound fraction contains all major proteins found in the extracted CWP (Fig. 4E). The void fraction shows slight background staining at the points of the major bands which is possibly due to incomplete binding. There are no enhanced bands present in the void fraction which are correspondingly absent in the bound fraction. Such results would indicate lack of recognition.

Figure 4D shows the protein profile obtained from cell wall protein extracted from the epicotyl growing region (apical 5–7 mm). Presumably, if there are ionically bound cell wall proteins involved in growth promotion they may be present in greater amounts in the growing regions. Comparisons of lanes D and E show no such enhancements. There are, however, some high mol w bands which are absent in the apical CWP.

A final qualitative test of the reactivity between CWP and the antibodies was accomplished through Western blot analysis. Nonspecific binding was prevented through several treatments with 3% casein + 1% goat serum. Figure 5 shows the developed blot. Arrows indicate four possible bands of nonantigenic protein (32.5, 24, 16.5, 16 kD). Two uneven bands at the top of the blot are artifacts due to lack of contact between the nitrocellulose and the gel. This assay confirmed that the antibodies recognized the majority of the CWP.

In Vitro Extension ('Creep') Assays. A further possible explanation for the lack of antibody effect on growth was that the metabolic processes of the cell were able to overcome inhibition. For this reason, *in vitro* extension was chosen to test the effects of antibodies on nonliving tissue. Frozen/thawed segments under constant stress will extend if placed in acidic solutions. The time course of this extension consists of a rapid phase and a slower

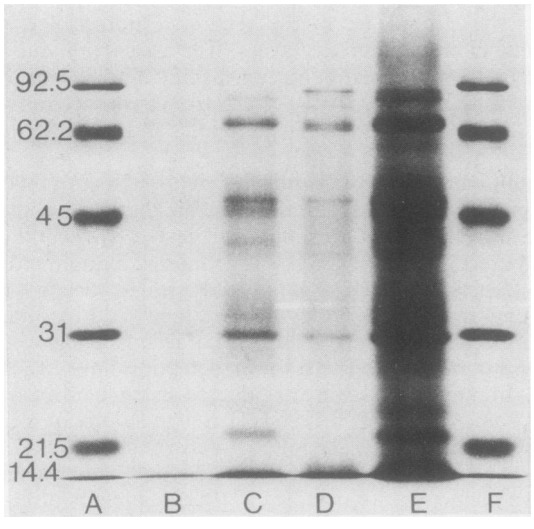

FIG. 4. SDS-PAGE of cell wall proteins. Lanes A, F, mol wt standards (20 μg); lane B, affinity column void fraction (20 μg); lane C, affinity column bound fraction (40 μg); lane D, cell wall protein from the apical (growing) region (20 μg); lane E, cell wall protein from the total epicotyl (50 μg). Gel stained with Coomassie blue R-250. Positions of markers indicated in kD.

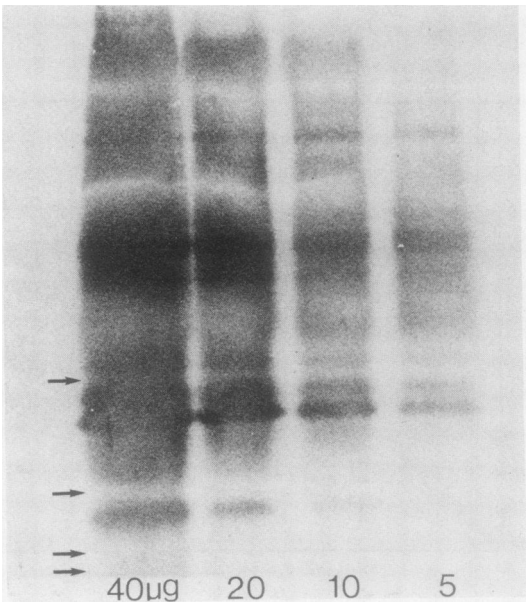

FIG. 5. Western blot of progressive dilutions of total epicotyl cell wall protein. Arrows indicate bands of nonantigenic proteins (32.5, 24, 16.5, 16 kD). Blot probed with 1:250 dilution of primary antiserum and 1:500 dilution of HRP-goat anti-rabbit IgG. Blot developed with 4-chloro-1-naphthol and H_2O_2.

phase, the latter of which may be under enzymic control (9). Results from creep experiments are presented in Table 1 and typical extension curves are seen in Figure 6. All pretreatments were made to frozen/thawed growing region segments except that perfusion pretreatments were carried out with living tissue which was then frozen.

Pretreatment of segments with immune IgG or $F(a,b)_2$ fragments (0.5 mg/ml; 2 h) did not reduce the extension rate. Pretreatments with boiling water (15 s) or protease (1 mg/ml; 3 h) eliminated the slower portion of creep. The pretreatment with boiling water decreased both the rapid and slower portions of creep whereas protease treatment eliminated only the slower phase. Addition of cell wall protein (1 mg/ml; 2 h) to untreated segments did not increase their extension rate nor did it serve to restore extension in protease pretreated segments.

Perfusion of the apoplast of living tissue with 50 mM LiCl removed proteins from the tissue (data not shown) but did not cause a reduction in the rate of extension. Perfusions of tissue were made using other salts (*i.e.*, $CaCl_2$, $LaCl_3$), but the results were inconsistent (data not shown). A more vigorous attempt to remove cell wall proteins was made via 3 M LiCl extraction.

Table I. *In vitro Extension Rates of Pea Segments*

Extension rates obtained after various pretreatments. Frozen/thawed segments placed on a constant stress extensometer for 45 min in 50 mM Hepes-KOH (pH 6.8) and changed to 50 mM Na acetate (pH 4.5) for 135 min. Increase in extension was calculated as extension rate at 175 min minus extension rate at 40 min divided by initial length.

Pretreatement	*n*	Increase in Extension Rate		Curve Type[a]
		%/h	SE	
Control	40	1.88	(0.15)	A
Immune IgG	8	2.45	(0.50)	A
Immune $F(a,b)_2$	8	1.53	(0.34)	A
50 mM LiCl perfusion[b]	10	2.08	(0.23)	A
3 M LiCl extraction	24	1.42	(0.17)	A
Cell wall protein	26	1.06	(0.23)	A
Protease	15	−0.07	(0.12)[c]	B
Protease then CWP	16	−0.24	(0.07)[c]	B
Boiling H_2O	8	−0.17	(0.07)[c]	C

[a] Curve types presented in Figure 6. [b] Pretreatment made with living tissue. [c] Significant difference at $p < 0.05$ from control values.

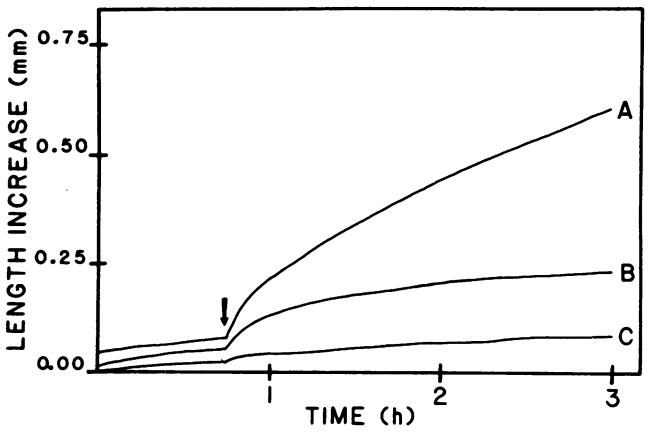

FIG. 6. Typical *in vitro* extension curves. A, Curve showing both rapid and slower phases of extension; B, extension curve showing a smaller rapid phase; C, extension curve showing little extension after treatment. Arrow indicates time at which pH was changed from 6.8 to 4.5.

Frozen/thawed tissue was incubated in 3 M LiCl for several hours with constant shaking and then washed to remove excess salt. The extraction procedure had no effect on creep rates.

DISCUSSION

This investigation has shown that antibodies raised against CPW extracted with 3M LiCl do not affect growth or *in vitro* extension. The antibodies penetrated the segment tissue and recognized the majority of the cell wall proteins. Furthermore, the activity associated with *in vitro* extension was not removed from segments with salt solutions.

Several possibilities exist to explain the results. First, the protein(s) involved in wall loosening in peas may not be ionically bound to the cell wall. The bonding may be covalent. In a recent study of cell wall enzymes, Nagahashi and Seibles (26) found that >80% of α-mannosidase activity remained bound to potato tuber cell walls after 3 M LiCl treatment. They proposed that an isozyme of α-mannosidase was covalently bound to the walls. Additionally, β-galactosidase, acid phosphatase and β-xylosidase were also found to be retained by the cell walls in substantial amounts (27.5, 27.4, and 11.4%, respectively). Similar results were also obtained with corn root and apple cell walls. Their work suggests that numerous enzymes may be covalently bound to the wall.

A second possibility is that the antibodies were too large to enter the wall matrix. Also, if proteins were sufficiently large, they might not escape from the entanglements in the intact cell wall. Pore sizes have not been measured in pea epicotyl walls, but bean hypocotyl walls were shown by gel filtration chromatography to admit proteins as large as 60 kD (33). Carpita *et al.* (6) found substantially smaller pores, 35 to 52 Å diameter (about 17 kD cutoff), in other plant material using a cell-collapse technique.

If pea walls have similarly small pores, then antibodies would not easily penetrate them. X-ray crystallography indicates an arm to arm distance of 146 Å for IgG (18) and dimensions of 30 × 40 × 50 Å for F(a,b) fragments (29). Fluorescence microscopy in this study showed that whole IgG can penetrate into tissue segments, but it is possible that the antibodies moved along a film of water on the surface of the wall, and did not penetrate the wall. Detailed electron microscopy would be needed to establish this point. On the other hand, there are a few reports which suggest that antibodies may penetrate plant tissues and exert physiological effects (12, 16, 19, 22). Moreover, relatively large proteins have been detected in the perfusate from pea epicotyls (25). These results suggest that wall pore sizes may be large enough to allow the required movement of large macromolecules.

The third explanation may be that of synergistic effects. Because the antibodies were raised against a broad range of proteins, they may affect proteins which inhibit growth as well as those which enhance growth. Fractionation of the protein preparation in the manner of Nevins *et al.* (27) could help to sort out the various components of the CWP.

The results from this study suggest that ionically bound wall proteins are not involved in wall loosening in pea epicotyls. Quite different results were obtained with corn coleoptiles, using a similar antibody approach (16, 27). Dicots and monocots are known to differ in wall composition and structure (reviewed in Carpita [5]), so it is possible that proteins with different characteristics mediate wall loosening and growth in the two cases. In peas the protein(s) responsible for wall loosening may be: (a) readily soluble in aqueous solutions (however, this is doubtful in light of the perfusion experiments in this study and in that by Morrow and Jones [25]); (b) covalently bound or thoroughly entangled in the wall; or (c) not present in the wall at all. Thus it would seem that the autolytic enzymes in pea walls which are

extractable with 3 M LiCl (2) may not be closely associated with cell enlargement.

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