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β-D-Glucan Antibodies Inhibit Auxin-Induced Cell Elongation and Changes in the Cell Wall of *Zea* Coleoptile Segments[1]

Takayuki Hoson and Donald J. Nevins*

Department of Biology, Faculty of Science, Osaka City University, Sumiyoshi-ku, Osaka 558, Japan (T.H.); *and Department of Vegetable Crops, University of California, Davis, California 95616* (D.J.N.)

ABSTRACT

Antiserum was raised against the *Avena sativa* L. caryopsis β-D-glucan fraction with an average molecular weight of 1.5 × 10⁴. Polyclonal antibodies recovered from the serum after Protein A-Sepharose column chromatography precipitated when cross-reacted with high molecular weight (1→3), (1→4)-β-D-glucans. These antibodies were effective in suppression of cell wall autohydrolytic reactions and auxin-induced decreases in noncellulosic glucose content of the cell wall of maize (*Zea mays* L.) coleoptiles. The results indicate antibody-mediated interference with *in situ* β-D-glucan degradation. The antibodies at a concentration of 200 micrograms per milliliter also suppress auxin-induced elongation by about 40% and cell wall loosening (measured by the minimum stress-relaxation time of the segments) of *Zea* coleoptiles. The suppression of elongation by antibodies was imposed without a lag period. Auxin-induced elongation, cell wall loosening, and chemical changes in the cell walls were near the levels of control tissues when segments were subjected to antibody preparation precipitated by a pretreatment with *Avena* caryopsis β-D-glucans. These results support the idea that the degradation of (1→3), (1→4)-β-D-glucans by cell wall enzymes is associated with the cell wall loosening responsible for auxin-induced elongation.

Noncellulosic β-D-glucans are recognized as components of the cell walls of the Gramineae (24, 28). These polysaccharides are apparently absent in the cell walls of *Zea* embryos, but the content increases rapidly with coleoptile growth, and then declines after elongation has subsided. The transient nature of the β-D-glucans is consistent with a developmental role (3, 18). A decrease in β-D-glucan content is also observed in walls of excised *Avena* coleoptile segments undergoing auxin-induced elongation (16, 17, 26). Similar modification of β-D-glucans has been reported during auxin-induced elongation in barley (27) and rice (34) coleoptiles. Correlations exist between auxin-induced decreases in the β-D-glucan content, changes in the mechanical properties of the cell walls (cell wall loosening), and cell elongation (26). These observations suggest a role for β-D-glucans in regulation of elongation growth.

The relationship between auxin-induced elongation and the degradation of β-D-glucans has been examined by imposing conditions which modify growth and/or interfere with normal glucan metabolism of *Avena* coleoptile segments. For example, auxin-induced decreases in β-D-glucan content have been observed in segments in the presence of 0.2 M mannitol solution, a concentration which imposes an osmotic equilibrium and suppresses auxin-induced elongation (17, 26). A decrease in the β-D-glucan content is therefore not necessarily a consequence of auxin-induced elongation, but it remains unclear whether the former is a cause of the latter. The reports that exo-(1→3)-β-glucanase derived from *Sclerotinia libertiana* induces elongation growth of *Avena* coleoptiles are also consistent with the concept that modification of β-D-glucan metabolism may facilitate elongation (19, 29, 32). The action of *Sclerotinia* glucanase is, however, unique in that it is the only enzyme that is known to induce growth among the diverse glucanases capable of degrading β-D-glucans. Nojirimycin, an inhibitor of exo-(1→3)-β-glucanase and β-glucosidase, inhibits auxin-induced elongation and prevents the decrease in β-D-glucan content (22, 26). While inhibition conforms with the concept that β-D-glucan degradation is associated with cell elongation, the effect of other modifiers of β-D-glucan degradation on the elongation process should be examined. Conventional enzyme inhibitors often lack specificity.

Antibodies raised against β-D-glucans or glucanases appear to offer binding specificity. In this study, we examined the effect of β-D-glucan antibodies on auxin-induced elongation and measured parameters in the cell wall of *Zea* coleoptile segments. Antibodies found to be specific inhibitors of β-D-glucan degradation also suppressed auxin-induced elongation and cell wall loosening.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. B73 × Mo17) seeds were soaked in running tap water for 24 h at 30°C, then grown in vermiculite saturated with water at 26°C in the dark for 3 d. Coleoptiles of 30 to 35 mm were selected, and first leaves were removed.

β-Glucan Preparation

Avena caryopsis β-D-glucan (provided by Quaker Oats, Inc.) was purified as described previously (14). The β-D-glucans contained 2.8% (w/w) proteins. The glucans were dissolved

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in 10 mM sodium phosphate buffer (pH 7.0), then applied to a hydroxylapatite column (Bio-Gel HTP, 1.2 × 5 cm), equilibrated with the same buffer. No protein was detected in column eluate. After precipitation by ethanol, the recovered β-D-glucans were dissolved in water, then applied to a Bio-Gel A-1.5 m or a A-50 m column (1.8 × 40 cm), preequilibrated with water. One mL fractions were collected, and the sugar content was measured. The average mol wt was estimated at 3×10^5 based on elution behavior from Bio-Gel A1.5 m (Fig. 1) or $>2 \times 10^6$ based on A-50 m chromatography (data not shown). A major sugar component purified by Bio-Gel A1.5 m chromatography (fractions 28–47) was collected. After repeated freeze and thaw cycles, the β-D-glucans were solubilized and examined with the same column. Part of the β-D-glucan derived from this treatment constituted a fraction with an average mol wt of 1.5×10^4 (Fig. 1). Fractions 61 to 75 were used to generate antibodies as follows.

Preparation of Antibodies

New Zealand white rabbits were administered, via intramuscular injection, with low mol wt β-D-glucan emulsified with an equal column of Freund's incomplete adjuvant (Difco Laboratories). A schedule of weekly injections was employed. Immunization dosage was initiated at 0.25 mg, increased to 0.5 and 1 mg at second and third injection, and thereafter maintained at 1.5 mg in subsequent injections. After 8 weeks, the rabbits were bled via the auricular artery at 2 week intervals.

The blood was maintained at room temperature for 2 h, and the clot was removed. The serum was centrifuged at 750 g for 20 min, then applied to a Protein A-Sepharose CL-4B column (Pharmacia, 1 × 2 cm), equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). The adsorbed immunoglobulins were eluted with 1 N acetic acid containing 0.15 M NaCl, then neutralized with NaOH. This fraction was dialyzed against PBS[2] and stored in capped vials at −20°C. PIS collected before the first antigen injection was similarly purified and stored.

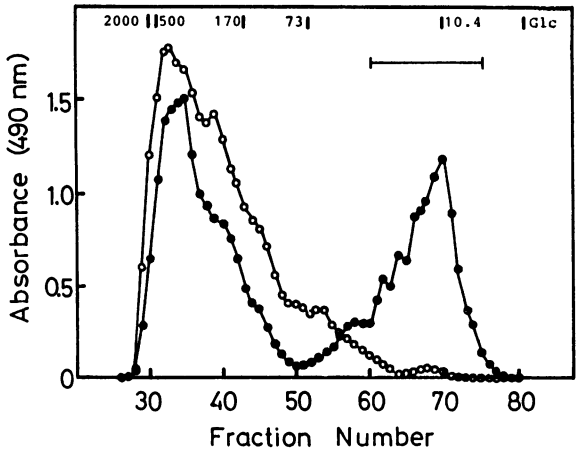

Figure 1. Gel filtration of *Avena* caryopsis β-D-glucans. The glucans, after hydroxylapatite column purification, were applied to a Bio-Gel A-1.5 m column (1.8 × 40 cm). Fractions of 1 mL were collected, and sugar content was measured (○). The major peak (fractions 28–47) was collected, then repeatedly frozen and thawed. These retrograded glucans were reexamined on the same column (●). High mol wt glucans thus obtained (fractions 28–50) were used for the precipitation assay and in antibody pretreatments. The low mol wt glucans (fractions 61–75) were used as the antigens for immunizing the rabbit. Vertical bars denote the elution positions of mol wt standards (× 1000) and glucose.

Precipitation Assay

The precipitation analysis was carried out according to the method of Kaku *et al.* (12). Antibodies (70 μg) were mixed with different amounts of polysaccharides in 0.29 mL of PBS. The reaction mixtures were kept at 4°C for 72 h, the precipitate was collected by centrifugation, washed twice with PBS, dissolved in 0.1 N NaOH, and then used in assays. High and low mol wt β-D-glucans generated by freeze-thaw cycles were separated on a Bio-Gel A-1.5 m column (Fig. 1, fractions 28–50 and 61–75). Lichenan (from *Cetraria islandica*), *Laminarin* (from *Laminara digitata*), and carboxymethylcellulose (type 7M) were purchased from Sigma, United States Biochemical, and Hercules, respectively.

Autolysis Experiments

Coleoptiles selected as described above were homogenized in ice-cold 50 mM NaCl for 5 min using a Waring blender. The homogenate was transferred to a polypropylene mesh (105 μm pore size), then washed with cold 50 mM NaCl, acetone (−20°C), and 50 mM NaCl. Ten mg of cell wall samples were incubated in 10 mM citrate-sodium phosphate buffer (pH 5.6), with or without antibodies or PIS at room temperature. In some experiments, antibodies (200 μg) were incubated with 200 μg of high mol wt β-D-glucans at 4°C for 24 h, followed by removal of precipitated proteins by centrifugation (pretreated serum). After a 2 h incubation with a selected antibody preparation, the cell wall was recovered by centrifugation and washed twice with 50 mM NaCl. The cell wall subjected to antibody treatment was then suspended in the same buffer containing 0.02% NaN_3 without antibodies or PIS and incubated at 35°C. The wall suspension was centrifuged at appropriate intervals, the liquid medium removed and the cell wall pellet was resuspended in fresh buffer. After the final centrifugation the cell wall was dissolved in 72% (v/v) sulfuric acid. The sugar content of each supernatant sample and the acid-solubilized cell wall were determined.

Determination of Noncellulosic Glucose Content

Segments cultured for an appropriate time were fixed in boiling methanol. These segments were ground in a mortar, then washed three times each with cold water, acetone, and a methanol-chloroform mixture (1:1, v/v). The cell wall samples were treated with α-amylase (type I-A Sigma, from *Porcine* pancreas), and thoroughly washed with water. The samples were dried, then hydrolyzed in a capped tube with 2 N TFA at 121°C for 1 h. After the TFA had been removed by evaporation under an air stream at 50°C, the sugars were

[2] Abbreviations: PBS, sodium phosphate buffer; PIS, preimmune serum.

reduced with sodium borohydride, then acetylated with acetic anhydride at 121°C for 3 h. Acetylated alditols were assayed by gas chromatography (Hitachi 063) according to the method of Albersheim *et al.* (1).

Growth Measurements

Prior to antibody pretreatment, the cuticle of coleoptiles was abraded with a carborundum slurry by rubbing the surface between the thumb and forefinger. Subapical segments of 10 mm were excised and floated on 10 mM Mes-KOH buffer (pH 6.0), containing different concentrations of antibodies or PIS at 26°C in the dark. Antibodies dissolved in PBS were dialyzed first against PBS diluted to half the original concentration, then against 10 mM Mes buffer to prevent precipitation. After 2 h, IAA (final concentration, 10^{-5} M) was added to some culture dishes, and growth was monitored. The length of segments was measured with the aid of a binocular microscope (×10) equipped with an ocular micrometer. Each growth experiment was repeated three times.

Measurement of Mechanical Properties of the Cell Wall

Ten mm segments treated with and without auxin were subjected to boiling methanol. The tissue was rehydrated, and fixed between two clamps of a tensile tester (Tensilon RTM-25, Toyo Baldwin, Tokyo). The distance between the upper and lower clamps was initially 5 mm. Segments were extended by displacement of the lower clamp at a rate of 20 mm/min to produce a stress of 10 g. Stress behavior as a function of time was recorded at ms intervals and the minimum stress-relaxation time of cell walls was calculated by the method of Yamamoto *et al.* (33).

General Methods

The sugar content was determined by the phenol-sulfuric acid method (6) as glucose equivalents. Proteins were estimated using the Bio-Rad Protein Assay with bovine serum albumin employed as a standard.

RESULTS

Interaction of Antibodies with Glucans

Precipitation analysis was employed to determine antibody specificity. Antibodies were mixed with different glucans, and the extent of protein precipitation was evaluated, Of all the β-D-glucans examined, only high mol wt glucans from *Avena* caryopsis and lichenan were capable of precipitating significant amounts of protein (Fig. 2). The extent of precipitation appeared to reach a maximum in the presence of 100 μg of high mol wt *Avena* glucan, whereas 200 μg of lichenan was required to approach similar effectiveness. β-Glucans, such as starch or glycogen, were incapable of precipitating antibodies (data not shown). Neither high mol wt *Avena* glucan nor lichenan precipitated protein from the PIS. Thus, antibodies obtained were specific for (1→3), (1→4)-β-D-glucans.

A maximum of 7 μg of protein was precipitated upon reaction with β-D-glucan antibodies (in the presence of 70 μg of *Avena* caryopsis glucan) (Fig. 2). The data suggest that

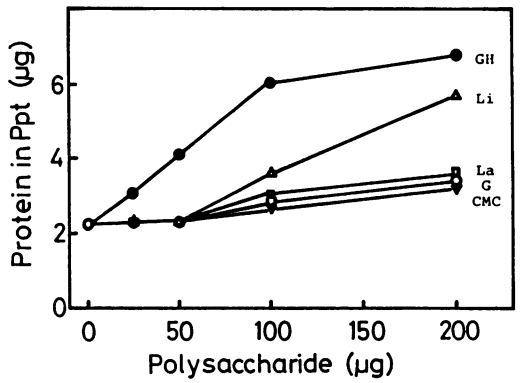

Figure 2. Quantitative precipitation of β-D-glucan antibodies with various β-D-glucans. Antibodies (70 μg) were incubated with different amounts of the glucans. The precipitates were assayed after 72 h. GH, High mol wt *Avena* caryopsis glucans; Li, lichenan; La, laminarin; G, low mol wt *Avena* glucan; CMC, carboxymethylcellulose. Values are means of three measurements.

nearly 10% of immunoglobulins contained in the antiserum were generated in response to β-D-glucans injected. The remaining immunoglobulins were likely generated against various natural antigens, since PIS contained nearly the same concentration of proteins as the serum of immunized rabbits.

Effect of Antibodies on β-D-glucan Degradation

Zea coleoptile cell walls incubated in buffer were capable of autohydrolysis of more than 12% of the component polysaccharides over a 36 h period (Fig. 3) (see also ref. 9). The β-D-glucan antibodies at 200 μg/mL suppressed autolysis by about 35% (Fig. 3). PIS and the antibodies pretreated with high mol wt *Avena* glucans had no effect on autolytic activity (Fig. 3).

Since more than 90% of the autolysis products of *Zea* cell walls were derived from (1→3), (1→4)-β-D-glucans (9), the results indicate that the β-D-glucan antibodies inhibit the *in vitro* degradation of β-D-glucans of *Zea* cell walls.

Effect of Antibodies on IAA Mediated Changes in Cell Walls

IAA at 10^{-5} M decreases the noncellulosic glucose content of Zea cell walls (Table I) similar to that observed in coleoptile segments of other species (16, 17, 26, 27, 34). The β-D-glucan antibodies at 200 μg/mL clearly suppressed auxin-induced changes in cell wall metabolism (Table I). Neither PIS nor the antibodies pretreated with high mol wt *Avena* glucans inhibited the auxin-induced decrease in noncellulosic glucose. These data also show that the β-D-glucan antibodies inhibit auxin-induced degradation of β-D-glucan *in vivo*.

Effect of Antibodies on Elongation and Cell Wall Loosening of Segments

The β-D-glucan antibodies at 100 μg/mL and above clearly suppressed auxin-induced elongation of abraded *Zea* coleoptile segments, without an apparent lag period (Fig. 4). Antibody pretreatment (400 μg/mL) suppressed elongation during

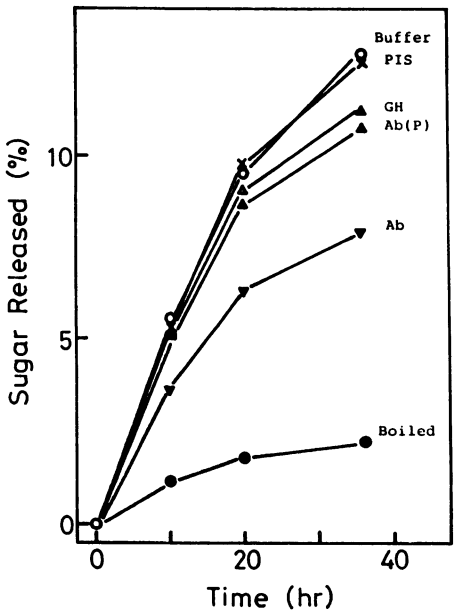

Figure 3. Autolytic reactions of isolated *Zea* cell wall as affected by β-D-glucan antibodies. The cell wall samples were preincubated in each medium for 2 h, then transferred to fresh buffer. The cell walls were incubated at 35°C and the amount of sugars released was determined. Concentration of PIS: 200 μg/mL. GH, High mol wt *Avena* glucans at 200 μg/mL; Ab, antibodies at 200 μg/mL: Ab(P), antibodies pretreated with high mol wt *Avena* glucans; boiled, the cell wall boiled for 5 min in methanol before incubation served as a control Autolytic activity is expressed as the ratio of the amount of sugar released to the total cell wall content (percentage). Values are means of three independent measurements, which never differed by more than 9% from the mean.

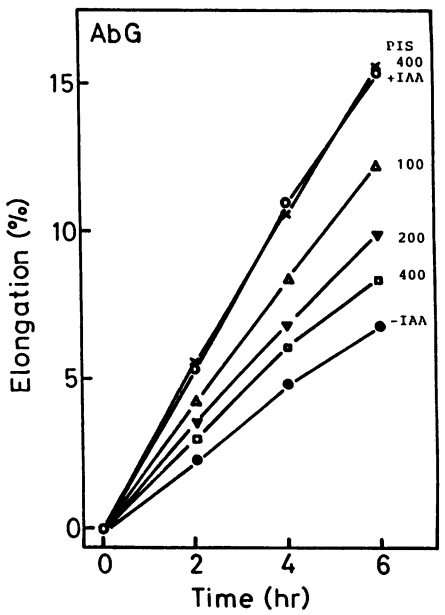

Figure 4. Elongation of *Zea* coleoptile segments as affected by β-D-glucan antibodies. Segments were floated on the buffer with (open symbols) or without (filled circle) 10^{-5} M IAA in media containing different concentrations (μg/mL) of the antibodies. (×), IAA plus PIS at 400 μg/mL. Elongation is expressed as the percentage increase in segment length. Values are means of three independent experiments (10 segments per treatment).

Table I. *Effect of Antibodies on Noncellulosic Glucose Content of Coleoptile Segments*

The cell walls, obtained from initial segments or segments treated for 4 h, were hydrolyzed in 2 N TFA, and the solubilized sugars were determined by GLC. Glucan antibody (pretreated), refers to antibody precipitated by high mol wt *Avena* glucans prior to the experiment. Concentrations of PIS, glucan, and antibody, 200 μg/mL. Values are means ± SE of three independent measurements.

	Glucose Content
	μg/segment
Initial	74.0 ± 0.29
Buffer	71.7 ± 0.50
IAA (10^{-5} M)	62.1 ± 0.52
IAA + PIS	64.5 ± 0.94
IAA + high mol wt glucan	61.6 ± 1.86
IAA + glucan antibody	69.1 ± 0.47
IAA + glucan antibody (pretreated)	61.5 ± 0.07

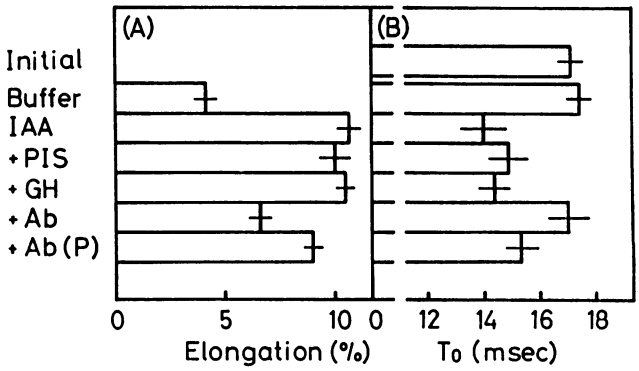

Figure 5. Effect of antibodies on elongation (A) and the minimum stress-relaxation time of the cell wall (B) of *Zea* segments. Segments were grown for 4 h. Concentration of PIS: 200 μg/mL. GH, High mol wt *Avena* β-D-glucans at 200 μg/mL: Ab, β-D-glucan antibodies at 200 μg/mL: Ab(P), antibodies pretreated with high mol wt *Avena* glucans. Values are means ± SE (n = 30 for A, 20 for B).

the subsequent 6 h incubation period by 48 and 28% in the presence and absence of IAA, respectively (data not shown). Antibodies preferentially affect auxin-induced elongation. PIS at 400 μg/mL did not influence cell elongation of coleoptile segments (Figs. 4 and 5A). Antibodies pretreated with high mol wt *Avena* glucans had only a small residual effect on elongation (Fig. 5A).

Stress-relaxation analysis was used to examine the effect of antibodies on auxin-induced changes in the mechanical properties of the cell walls. IAA at 10^{-5} M caused a decrease in the minimum stress-relaxation time of the cell wall, as reported previously (26, 27, 33). β-D-glucan antibodies at 200 μg/mL significantly inhibited the auxin-induced decrease in the stress-relaxation time (Fig. 5B). PIS had no effect. Inhibition of auxin-induced cell wall loosening was much less when antibodies were pretreated with high mol wt *Avena* glucans. Thus, β-D-glucan antibodies specifically suppressed both auxin-induced elongation and cell wall loosening.

DISCUSSION

Antibodies raised against *Avena* caryopsis β-D-glucans clearly suppress autolytic reactions of isolated cell walls (Fig. 3) and the IAA-induced decrease in noncellulosic glucose content (Table I) of *Zea* coleoptile segments. IAA-induced elongation (Fig. 4) and cell wall loosening (Fig. 5) of *Zea* coleoptile segments were also suppressed. These results strongly support the idea that the degradation of (1→3), (1→4)-β-D-glucans by wall associated enzymes mediates cell wall loosening responsible for auxin-induced cell elongation (9, 11, 26, 17).

Dissociation of the binding between the antibodies and β-D-glucans is expected during the fixation process in boiling methanol in preparation for stress-relaxation analysis. Thus, β-D-glucan antibodies appear to inhibit wall loosening events by associations during the incubation period and not by the formation of cross-links between glucan polymers. Antibodies raised against endo-glucanase obtained from *Zea* cell walls also suppressed β-D-glucan degradation and auxin-induced elongation of *Zea* coleoptile segments (data not shown). It is likely that the degradation of (1→3),(1→4)-β-D-glucans is inhibited when either the substrate (glucans) or the enzyme (endoglucanases) is subjected to antibody binding.

The specificity of β-D-glucan antibodies on β-D-glucan degradation, elongation, and cell wall loosening was examined physiologically using PIS and antibodies pretreated with *Avena* caryopsis β-D-glucans as controls. PIS did not affect auxin-induced β-D-glucan degradation (Fig. 3; Table I), cell elongation (Figs. 4 and 5), or cell wall loosening (Fig. 5). Antibodies precipitated by appropriate pretreatment also did not suppress autolytic reactions of isolated cell wall (Fig. 3) or IAA-induced decreases in noncellulosic glucose content of wall (Table I). Antibodies pretreated with β-D-glucan only slightly suppressed auxin-induced elongation and cell wall loosening of *Zea* coleoptiles (Figs. 4 and 5).

Of the β-D-glucans examined, only high mol wt glucans from *Avena* caryopsis and lichenan were capable of precipitating antibodies (Fig. 2). This observation confirms antibody specificity for (1→3), (→4)-β-D-glucans. Glucans are thought to form linear chains in which two or three consecutive (1→4)-linked glycosyl residues were separated by single (1→3)-linked glycosyl residues (20, 23). However, Kato and Nevins (14, 15) showed that β-D-glucans from *Zea* cell walls also contained intermittent contiguous sequences of three (1→3)-linkages that may confer a folded structure. There is the possibility that sequences of (1→3)-linked glucosyl residues, together with the adjacent (1→4)-linked glucosyl residues, constitute antigenic determinants.

β-D-glucans with an average mol wt of 1.5×10^4 used as the antigens did not precipitate significant amounts of antibodies (Fig. 2). We suggest that small glucan molecules may have less than three antibody-binding sites per molecule. Therefore, low mol wt glucans partially restore β-D-glucan degradation, elongation, and cell wall loosening, when they were added simultaneously with inhibitory antibodies (data not shown). The binding to β-D-glucans may inactivate antibodies even though precipitates are not formed.

Low mol wt β-D-glucans were generated by a procedure involving repeated freeze and thaw cycles. Huber and Nevins (9, 10) and Hatfield and Nevins (7) reported that similar sized β-D-glucans were products of *Zea* endoglucanase action. These results suggest that β-D-glucans are interrupted by occasional regions which are enzymically and/or physically fragile. Enzymic digestion of plant cell walls solubilizes rhamnogalacturonans (5) and xyloglucans (13) with an average mol wt of 1 to 2×10^4. Carbohydrate chains of this size appear to form a domain perhaps common to many matrix polysaccharides.

Structures of (1→3), (1→4)-β-D-glucans have been examined in the walls of *Avena* coleoptiles (7, 11, 23, 31), *Zea* coleoptiles (14, 15), and *Hordeum* endosperm (30). β-D-Glucans from *Avena* coleoptiles are similar to those of *Zea* coleoptiles. Moreover, β-D-glucan antibodies suppressed elongation of coleoptile segments of both *Avena* (data not shown) and *Zea* (Figs. 4 and 5). On the contrary, (1→3), (1→4)-β-D-glucans have not been detected in dicotyledons (20) and the antibodies did not influence auxin-induced elongation of stem segments of dicotyledons (data not shown). Thus, the mechanism of auxin regulation of cell wall extension may differ among taxa, probably due to the difference in the matrix polysaccharide complement.

The antibody-mediated suppression of elongation was not always complete (Figs. 4 and 5). One reason may be that the wall components other than β-D-glucans are also involved in auxin-induced elongation. We avoided the use of antibodies at higher concentration, since there would be a tendency to form aggregations. The concentrations utilized may account for incomplete suppression of elongation by antibodies.

β-D-Glucan antibodies clearly influenced cell wall metabolism (Fig. 3; Table I) and the mechanical properties of the cell walls (Fig. 5) as well as elongation growth (Figs. 4 and 5). These results indicate that antibodies can penetrate into the cell walls despite their high mol wt ($1.5–1.9 \times 10^5$). The results obtained in the present study support the idea (8) that the actual pore size of the cell walls is larger than that measured by a solute exclusion technique (4).

Lectins and antibodies, showing specific binding for sugar residues, could be important probes for the location and function of cell wall polysaccharides (25). Lectins have been used as an approach to clarify the mechanism of cell wall loosening (8) as well as to identify the location of cell wall polysaccharides (2, 25). To date, antibodies have primarily been used to localize certain cell wall components (21, 25). The present study represents the first attempt to exploit antibody specificity directed toward polysaccharides as a method to understand the mechanism of cell wall extension. The *in situ* location of β-D-glucans is under investigation using the β-D-glucan antibodies.

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